VARIABILITY IN CULTURED CELLS OF *CAPSICUM* SPP.

by

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ABBREVIATIONS

A	aggregated
ANOVA	analysis of variance
6-BAP	6-benzylaminopurine
B	brown
BSA	bovine serum albumin
ca.	approximately
cm.	centimetre(s)
$CHCL_3$	chloroform
CMV	culture mean value
CAS	culture average score
[ ]	concentration
cv.	cultivar
d.	day(s)
°C	degrees centigrade
DF	degrees of freedom
diam.	diameter
2,4-D	2,4-dichlorophenoxyacetic acid
DW	dry weight
E	einsteins
et al.	*et alia*
EtOH	ethanol
F	friable
Fn	fine
FW	fresh weight
g.	gram(s)
G	green
h.	hour(s)
HPLC	high performance liquid chromatography
IAA	indole-3-acetic acid
l.	litre(s)
log	logarithmic
LSD	least significant difference
MeOH	methanol
m.	metre(s), milli- (10^{-3})
µ	micro (10^{-6})
ml.	millilitre(s)
mg.	milligram(s)
min.	minutes
M	molar
MS	mean square
NAA	α-naphthaleneacetic acid
n.	nano- (10^{-9})
N.D	not detectable
no.	number
OD	optical density

O	orange
%	percent.
PAL	phenylalanine ammonia lyase
pH	negative log of the hydrogen ion concentration
psi	pounds per square inch
+/-	plus or minus
RGR	relative growth rate
rpm	revolutions per minute
s.d	standard deviation
SH	schenk and hildebrandt medium
SPCs	soluble phenolic compounds
spp.	species (plural)
SS	sum of squares
SS%	percentage sum of squares
TCC	total chlorophyll content
TPC	total protein content
VR	variance ratio
v/v	volume per volume (as percentage)
Wt	watts
w/v	weight per volume (as percentage)
wt	weight
W	white
xg	times the force of gravity
$\bar{x}$	mean
Y	yellow

CONTENTS

ABSTRACT

This thesis reports on experiments which investigate the nature and causes of variation in tissue cultures of *Capsicum* spp., and discusses the implications of these findings in relation to the control and exploitation of variation.

It was shown that single cells isolated from a suspension culture of *Capsicum frutescens* cv. Cayenne and cultured individually gave rise to clones initially of callus and then of suspended cells which exhibited considerable differences in morphological and metabolic characteristics. Some of these clones survived for a prolonged period in culture. Daughter cultures from one of these clones, AE2, were maintained in different culture conditions to determine the nature of its stability. The results showed that the daughter cultures derived from the clone remained stable as callus, whereas suspensions or immobilized suspension cultures differed in growth, morphology and metabolism showing a major effect of the culture condition. In addition, suspended and especially immobilized cultures produced considerably greater amounts of phenolic compounds than the callus. The reasons for the differences and instability in the cultures are discussed.

The effect of medium constituents and explant origin on the generation of variation was also investigated. Suspension cultures of clone AE2 were found to be incapable of growing successfully in medium without, or with markedly reduced amounts of, sucrose, nitrate and phosphate, whereas they were unaffected by substantial changes in the level of 2,4-D and pH. Differences in PAL activity were attributed more to the morphological state of the cultures itself than to changes in the concentration of medium constituents. The results also showed that establishing callus cultures from different parts of the plant or from plants of different ages of *Capsicum frutescens* cv. Cayenne did not affect the relative growth rate over a prolonged period in culture. However, differences were found in the appearance and growth of callus cultures derived from different *Capsicum* 'species', and the implications of these genotypic differences were considered.

Attempts to regenerate callus of clone AE2 were unsuccessful, however, regeneration was achieved using younger material particularly from callus derived from hypocotyl tissue suggesting that there was a loss in regenerative potential with increasing time in culture.

CHAPTER ONE

INTRODUCTION

Capsicum, a model plant for studies in tissue culture

The genus *Capsicum* of the family *Solanaceae* contains around thirty wild and domesticated species of chilli pepper, as well as many species of bell pepper (Pickersgill 1987). Their dried pungent fruits (chillies) manufactured into a powdered form (red or cayenne pepper) are widely used as a spice (Purseglove 1968). The secondary compounds responsible for the hot taste in the fruit are capsaicinoids of which capsaicin is the major component (Purseglove 1968). Capsaicin obtained in the crude form, as an oleoresin, can cost up to £350 per kilogram, whereas in the pure form it costs £84 per gram (Sigma Ltd 1989). In fact, capsaicin is only one of a large number of secondary compounds from the plant kingdom used in the pharmaceutical, food, flavour and perfume industries (Fowler 1983).

The use of plant tissue culture has provided a further dimension to the understanding and exploitation of secondary metabolite production (Dougall 1981, Curtin 1983, Collin 1987). One advantage is that it provides an environment in which several of the developmental and environmental constraints known to affect the plant can be controlled (Fowler 1983). Also under such conditions many aspects of the regulation of secondary pathways and product synthesis can be investigated (Yeoman 1987). Furthermore, cultures can be identified which, when manipulated, can produce greater amounts of secondary products than the donor plant (Tabata and Fujita 1985, Zenk *et al.* 1977, Curtin 1983). For example, the production of capsaicin (8-methyl-N-vanillyl-6-nonenamide) in tissue cultures of *Capsicum frutescens* has been increased substantially by immobilization, nutrient limitation and precursor feeding (Yeoman *et al.* 1980, Lindsey 1985, Lindsey 1986). From findings such as these the exploitation of plant cell cultures as a source of secondary products can be envisaged. One compound, shikonin, a dye with anti-bacterial and anti-inflammatory properties, is now produced commercially using high yielding cultures of *Lithospermum erythrorhizon*, and a second, berberine, from cultures of *Coptis japonica* is anticipated (Curtin 1983). However, although many plant species are high yielding in tissue culture their exploitation is hindered by instability. The drop in secondary product with increasing time in culture has been well documented, and manipulations such as cloning or re-cloning are needed to retrieve high yielding cultures (Dougall *et al.* 1980, Deus-Neumann and Zenk 1984).

Along with the exploitation of secondary products, the rapid and exciting advances which have been made in the genetic manipulation of plants would not have been possible without the use of tissue culture techniques (Potrykus *et al.* 1987, Schweiger *et al.* 1987). Furthermore, many species of plants can be regenerated back from culture, the number of which has risen exponentially over the past decade (Evans *et al.* 1981, Vasil 1986). In a genetic context this can permit the evaluation of *in vitro* genome manipulation in a mature plant, which is the main driving force responsible for the integration of tissue culture techniques into plant improvement (Evans *et al.* 1984, Scowcroft *et al.* 1985, Ryan *et al.* 1987). Tissue culture may also provide the opportunity for long term germplasm storage through minimal growth or cryopreservation (Withers 1986). However, manipulations like these are only successful if the cell material is both amenable to and stable in culture. In this respect, the outcome and potential of genetic manipulation is often hindered by the inability to regenerate cells back from culture due mainly to the culture-induced variation which causes the decline in cell totipotency (Karp and Maddock 1984).

It is now widely recognised that variation is ubiquitous to plant tissue cultures and hampers experimental research (Skirvin 1978, Gould 1986). Nevertheless, in some cases the emergence of variant cell types can be exploited to a practical gain in, for example, the isolation of high yielding cell lines by cloning ie; the shikonin producing cultures of *Lithospermum* (Curtin 1983). However, such examples prove to be exceptions to the rule and are likely to remain so until a better understanding of the factors regulating variation is obtained.

Variation in tissue culture

Even when the excision and transfer of plant parts to a defined growth medium is carefully controlled, a heterogeneous callus culture will result (Yeoman and Forche 1980). This is not surprising because the explant is usually heterogeneous with respect to cell type and gives rise to a so-called dedifferentiated population of cells in response to the culture conditions (Lindsey and Yeoman 1985). Furthermore, the initiation of callus in an alien culture environment may disturb the genetic integrity of the cells and hence the expression of morphological and metabolic characteristics, further increasing the heterogeneity of the callus culture.

The genetic origin of variation

Chromosomal changes have been reported in cultures from a wide range of plant species (for reviews see Sunderland 1977, Bayliss 1980, Gould 1986). These include aneuploidy and polyploidy, and also structural changes in the chromosome such as point mutations and gene transpositions (Bayliss 1980). Point mutations or irreversible changes in gene expression have been identified and selected for by their resistance to toxic substances, for example, antimetabolites, amino acids and herbicides (Dix 1986). Epigenetic changes have also been found to exist in culture (Meins 1986). This type of alteration also occurs at a single gene locus, however it is distinct from a point mutation as it is reversible and can be influenced by the physiological and developmental state of the cells (Meins and Binns 1977).

The identification of genetic change has been mainly carried out with fixed and stained material, and involves the direct examination of chromosome number and structure within individual cells of a culture population by using light microscopy or microdensitometry (Evans and Reed 1981). However, this procedure can be tedious and complicated, especially in tissue in which the cells have a high chromosome number and is limited in value as only cells in mitosis can be examined which only represents around 1% of the total population (Orton 1983). The recently developed technique of flow cytometry can overcome this problem to some extent as it can be used to determine the DNA content of each cell within a culture at any one time, although small changes in DNA content cannot be detected (Kruth 1982, Brown 1984). Many of the genetic changes present in cultured cells can also be identified as heritable mutations in the progeny of plants regenerated from tissue culture. This phenomenon, termed somaclonal variation, allows the application of a Mendelian perspective to the variation that is generated in tissue culture (Larkin and Scowcroft 1981). Such procedures have shown culture induced variation by revealing alterations in the chromosome number (Lee and Phillips 1988), activation of transposable elements (Peschke *et al.* 1987) and specific gene changes (Dennis *et al.* 1987) in the regenerated plants.

Types of variation in secondary metabolism

Variation in the ability to accumulate secondary metabolites has been reported among and within cultures of many plant species (Dougall, 1985). Creche *et al.* 1987 have shown differences in alkaloid production among individual cultures of *Choisya ternata*, while Constabel *et al.* (1981) revealed qualitative differences in alkaloid production among seventy-six *Catharanthus roseus* cultures. Many reports concerning variation in secondary metabolite production have also been centred around the differences among cells of a culture with the intention of isolating and establishing high yielding cell lines (Dougall *et al.* 1980, Berlin *et al.* 1981, Deus-Neumann and Zenk 1984). Cell lines of tobacco isolated from a single culture were shown to vary considerably in their nicotine contents (Tabata and Hiraoka 1976, Ogino *et al.* 1978). Similarily, Ellis (1985) has shown differences in the levels of rosmarinic acid among cultures derived from individual cells of *Anchusa officinalis*, and Hall and Yeoman (1987) have shown the anthocyanin content of *Catharanthus roseus* cultures isolated from single cells to vary 30 fold.

Another way of observing variation in secondary metabolite production is by using microspectrophotometric or microdensitometric techniques to assess the intracellular concentration of a designated product within a single cell. This procedure is a better way to measure variation as it determines the differences among cells at one time, therefore eliminating any temporal effects that can be caused by the cloning procedure. For example, microspectrophotometric analysis of the intracellular concentration of rosmarinic acid in single cells of an *Anchusa* culture has revealed great differences (Chaprin and Ellis 1984). Similarly, microdensitometric determinations of individual cells has shown variation in the intra-cultural anthocyanin content of a *Catharanthus roseus* culture (Hall and Yeoman 1987).

Types of variation in growth and morphology

Cultures isolated from single cells or by cloning small aggregates have been shown to exhibit a considerable degree of variation in their morphology. Chaturvedi and Mitra (1975) have described two subclones of *Citrus grandis* callus grown under identical culture conditions and showed that one consistently formed numerous embryoids while the other formed shoots. Selby and Collin (1976) in a study with twenty subclones of the cultures of

each of three onion varieties found great differences in friability, sliminess and growth rate. Similarily, three cell lines differing from one another in growth rate and morphology were isolated from an *Atropa belladonna* culture (Davey *et al.* 1971).

Factors affecting variation in tissue culture

The explant and donor plant

There is evidence to suggest that the type of explanted cells can influence variation in culture (Lindsey and Yeoman 1985). The heterogeneous nature of cells within the explant has been demonstrated by Torrey (1965) who found that segments of pea seedling roots contained two distinct populations (diploid and tetraploid) of cells which differed in growth requirements. Similarly, cultures from pith explants of tobacco were shown to differ in their requirements for cytokinin (Meins *et al.* 1980). Snijman *et al.* (1977) also found that the variability in the growth of cultured cells of tobacco pith explants could be related to differences in the endogenous levels of growth regulators.

Explants taken from different parts of the same plant (Arnison and Boll 1975, Wernicke and Brettel 1980), from different plants of a group (Zenk *et al.* 1977, Kinnersley and Dougall 1980) or from plants of different ages or species (Ketel *et al.* 1985, Mikami and Kinoshita 1988) have been shown to affect the morphology and metabolism of resulting cell cultures. However, other reports have provided evidence suggesting that there is no positive correlation with respect to the effect of the explant on culture (Speake *et al.* 1964, Dhoot and Henshaw 1977). Mantell and Smith (1983) proposed, with reference to metabolic activity, that all the necessary genetic and physiological potential for secondary metabolism is present in an isolated totipotent cell, and that cultured cells, irrespective of the part of the plant from which they excised, can be expected to yield similar secondary metabolites when held under stable cultural conditions. Although the effect of the explant and its origin cannot be ignored, evidence indicates that the great majority of variation develops during the period of tissue culture. Lorz and Scowcroft (1983) carried out experiments using the semi-dominant aurea mutant, sulphur, of tobacco, to descriminate between pre-existing and culture induced variation, and concluded that the occurrence of the genetic event that caused leaf yellowing in the regenerated plants was significantly enhanced during tissue culture.

Culture-induced variation

The growth of callus and cell suspension cultures, as measured by increases in, for example, fresh weight, dry weight or cell number against time, takes the generalised form of a sigmoidal curve (Street 1977, Aitchison *et al.* 1977). During one of these culture cycles the cells progress through a lag, exponential and stationary phase where noticeable changes in the structure, physiology and metabolism of the cells are observed (Lindsey and Yeoman 1985). At each subculture a small proportion of the cell population is transferred to fresh medium. The act of subculture probably contributes significantly to the variation observed in tissue culture as an unconscious selection pressure is being applied to the culture. Usually conditions for cell culture are chosen as being conducive to maximum growth rate, therefore creating conditions for the selective accumulation of cells which are capable for rapid growth (Lindsey and Yeoman 1985). It has been shown that the faster growing cell types contain a greater number of genetic irregularities in cultures of carrot (Bayliss 1980). From this it could be assumed that although being highly practical for experimental purposes, the standard growth conditions cause greater genetic variation among the cells of the culture. On the other hand Singh *et al.* (1975) showed that the proportion of normal 4-chromosome cells increased rapidly compared to the 16-chromosome cells in *Haplopappus gracilis* cultures over a 90 day period. These two studies show how the selection of a genetically distinct cell line can result in its predominance in a cell population.

Growth habit has also been shown to be related to the genotype and phenotype of cells. Orton (1980) compared callus types of an interspecific hybrid of *Hordeum* with respect to chromosomal constitution and regenerative capacity. Hard, nodular and slow growing calli were relatively stable in chromosome number and retained the capacity to regenerate shoots, whereas friable and fast growing calli exhibited a high proportion of chromosomally aberrent cells with a poor regeneration capacity. The length of time cells are left in culture is also known to affect the frequency of chromosomal changes (Bayliss 1980, Meins 1983). Most documented examples of time induced variation describe loss of totipotency, where the most logical explanation for this is the loss or mutation of genes which are essential for regeneration (Scowcroft 1984). In regenerated oats the frequency of cytogenetically abnormal plants increased dramatically with increasing time in culture, where

abnormalities included chromosome breakage, loss, interchanges and aneuploids (M^{c}Coy *et al.* 1982). A decline in the biosynthetic potential of cultured cells with time in culture has also been reported (Dougall 1987). Subclones of carrot showed a differential decline in anthocyanin production over a period of six subcultures (Dougall *et al.* 1980), and a drop in the ajmalicine, serpentine and secologanin contents of cloned *Catharanthus roseus* cultures has also been reported (Deus-Neumann and Zenk 1984).

Environmentally-induced variation

Temperature, light, viscosity, osmolarity and pH of the culture medium, and agitation rate are all controllable physical aspects of the culture environment and are all known to affect the cell division cycle of cells of many plant species in culture (Martin 1980, Gould 1986). Both light and temperature have been shown to influence the accumulation of secondary metabolites in cultures. Knobloch *et al.* (1982) have shown that the activity of the enzyme phenylalanine ammonia lyase is directly influenced by the presence or absence of light, while this environmental factor has also been shown to increase the polyphenol content of cultures of Paul's Scarlet Rose (Davies 1972). With respect to temperature, Nettleship and Slaytor (1974) showed that optimal growth of callus occurred at 30°C in *Peganum* cultures while maximum alkaloid production was attained at 25°C with levels of production decreasing rapidly as the temperature increased.

Alterations in medium constituents have also been shown to affect the morphology and metabolism of cultured cells (Mantell and Smith 1983, Lindsey and Yeoman 1985, Collin 1987). Growth regulators have been shown to be effective in stimulating or inhibiting secondary product synthesis (for reviews see Staba 1980, Mantell and Smith 1983). The auxins, such as 2,4-D, appear to have the greatest influence on the biosynthetic activity of cultures (Dougall 1980). Tabata and Hiraoka (1976) have shown that nicotine production in tobacco cultures is inhibited by the presence of 2,4-D, although another auxin, IAA, caused tobacco cultures to produce nicotine as well as anatabine and anabasine (Furuya *et al.* 1971). Some reports suggest that 2,4-D causes chromosome instabilty in cultures, but most results point to this as being a secondary effect related to the promotion of rapid, disorganised growth, rather than direct mutagenic action (Bayliss 1980).

Other constituents of the medium are known to affect the biosynthetic activity of cultured cells. Nutrients, particularly phosphate, ammonia and nitrate, as well as carbon sources such as sucrose, are known to affect the secondary metabolism of cultured cells (Dougall 1980). Knobloch and Berlin (1983) have shown that a rapid accumulation of secondary compounds occurs when cells of *Catharanthus roseus* were grown in a medium devoid of phosphate, a result that was also demonstrated with carrot (Dougall and Weyrauch 1980) and sycamore cultures (Phillips and Henshaw 1977). Furthermore, media with reduced levels of ammonia and nitrate also appear to stimulate biosynthetic activity of cultured cells (Phillips and Henshaw 1977). Lindsey (1985) has shown that a reduction in the concentration of nitrate and phosphate in the medium of *Capsicum frutescens* cultures causes a reduction in the incorporation of ^{14}C phenylalanine into protein and an increase into capsaicin. An increase in sucrose has been shown to stimulate phenolic synthesis and PAL activity in cultured sycamore cells (Westcott and Henshaw 1976, Phillips and Henshaw 1977) while a high sucrose concentration also stimulated alkaloid synthesis in *Catharanthus roseus* cultures (Knobloch *et al.* 1982).

Capsicum, a model plant for studies on variation in tissue culture

The genetical, morphological and metabolic variation in tissue culture, as well as the heritable and environmental factors that cause these changes, has been well documented in many plant species. However, little research on this subject has been carried out with tissue culture of the chilli pepper. As it has already been mentioned *Capsicum frutescens* cv. Cayenne has provided an ideal plant source for studies on the regulation and manipulation of secondary metabolism in tissue culture (Yeoman *et al.* 1980, Holden *et al.* 1987). Furthermore, capsaicin can be produced in manipulated cultures in amounts that suggest that it could be exploited on a commercial basis (Mavituna *et al.* 1987). However, these cultures are known to exhibit considerable variation in their biosynthetic capacity, as well as in metabolism, growth and morphology (Holden *et al.* 1987). For example, studies in this laboratory have demonstrated that callus clones developed from single cells of a suspension culture of *Capsicum frutescens* varied considerably in their capsaicin content (Lindsey and Yeoman 1985). Tissue cultures of *Capsicum* appear to harbour a considerable amount of variation; the incidence and causes of this have not yet been fully investigated.

AIMS AND OBJECTIVES

The aim of the work embodied in this thesis was to investigate the intrinsic and extrinsic factors that influence variation in cell cultures of *Capsicum* with a view to suggesting how they might be reduced or controlled. This aim can be broken down into the following objectives;

1. To characterise and compare the differences among clones isolated from single cells of a suspension culture so as to investigate the variation of the parent culture and to establish stable callus cultures.

2. To compare the differences among cultures derived from a stable callus clone maintained in a number of culture conditions so as to investigate the effects of different culture regimes on the generation of variation on the one hand and the maintenance of stability on the other.

3. To determine the role of medium constituents in the generation and occurence of variation in suspension cultures

4. To determine the role of explant origin in the generation of variation in callus culture

5. To develop a callus regeneration medium with a view to regenerating callus culture and assessing variation in the regenerated plant.

WORKING DEFINITIONS

- **Culture**, a population of cells maintained in controlled conditions.

- **Subculture**, the procedure by which a proportion of cells is taken at random from a culture and transferred to a new condition.

- **Clone**, cultured material that can be traced back to and derived from the mitotic divisions of a single cell.

- **Regime**, a controlled condition in which cultures are maintained, as in callus, suspension or immobilized suspension.

- **Sample**, a proportion of cells taken at random from a culture on which measurements are made.

- **Friable**, describes soft, breakable callus or highly dispersed suspension cultures.

- **Aggregated**, describes hard compact callus or suspension cultures.

- **Immobilized**, describes cells which have been entrapped in polyurethane foam blocks while in liquid culture.

- **Variegated**, describes cultures which have more than two types of coloured cells.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Plant material

Seeds of the following variety of plant, the major species under investigation, were obtained from McNair, Portobello, Edinburgh.

Capsicum frutescens Mill. cv. Cayenne

Seeds of the following plants were collected by and obtained as a kind gift from Dr. B. Pickersgill, Dept of Agricultural Botany, University of Reading.

Capsicum annuum

Capsicum baccatum

Capsicum chinense

Capsicum frutescens (wild type)

Seeds obtained from the above sources were sown in 10 cm. pots in a mixture of Fisons Levington potting compost and Perlite. These pots were placed in the Botany Dept. greenhouse (16h. day, 400Wt Mercury Vapour light source; 16°C. to 25°C. night temp depending on the season) where the seeds germinated. Watering was carried out as required to keep the compost moist. After six weeks the seedlings were transferred to 17.5cm pots in which they remained until they were required for experimental purposes. Explant excisions were taken at different times over the life cycle of the plant which lasted up to *ca.* 5 months depending on the species.

2.2 Tissue and cell culture

2.2.1 Media and growth regulator preparation

2.2.1.1 Standard growth medium

A supplemented Schenk and Hildebrandt medium (SH) (Schenk and Hildebrandt 1972) was used routinely as the standard growth medium for callus, suspension and immobilized suspension cultures. The medium used in experiments on the effect of medium components was based on this standard growth medium but contained different concentrations of 2,4-D, sucrose, nitrate and phosphate, and different pH levels, all of which are described in greater detail in Section 3.2. The medium used for regeneration was based on the standard growth medium but with different auxin and cytokinin growth regulators the types and concentrations of which are described in greater detail in Section 3.4.

The exact composition of the standard growth medium is given in Table 2.2.1. The standard growth medium was supplemented with 30g. l^{-1} Sucrose (BDH, Poole, Dorset), 0.5mg. l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1mg. l^{-1} Kinetin.

Stock solutions of macroelements, microelements, vitamins and growth regulators were prepared separately. Macroelements were prepared at 100 times the final concentration, dissolved in 1 litre distilled water, and stored at -40°C. When required 10ml aliquots of this stock were used for every litre of medium. Microelements, iron and vitamins were made up at 50 times the final concentration in 50ml. distilled water and stored at 4°C. When required 1ml. aliquots of this stock were used for every litre of medium. The growth regulators were added from stock solutions at a concentration of 1 mg ml^{-1}, the preparation of which is described in Section 2.2.1.3, and were stored at 4°C. After addition of all constituents the pH of the medium was adjusted to 5.8 using 0.1M. potassium hydroxide (KOH) or 0.1M. hydrochloric acid (HCL) before being made up to the correct concentration in a volumetric flask. Callus cultures were grown on solidified medium containing 6g l^{-1} agar (Bacteriological, Oxoid no.3, Oxoid Ltd).

Table 2.2.1

The constituents and concentration (mg. l^{-1}) of Schenk and Hildebrandt standard growth medium used in this investigation.

Constituents	Concentration in Media (mg. l^{-1})
	SH
Macroelements	
KNO_3	2500
$MgSO_4.7H_2O$	400
$NH_4H_2PO_4$	300
$CaCl_2.2H_2O$	200
Microelements	
$MnSO_4.4H_2O$	10
H_3BO_3	5.0
$ZnSO_4.7H_2O$	1.0
KI	1.0
$CuSO_4.5H_2O$	0.2
$CoCl_2.6H_2O$	0.1
$NaMoO_4.2H_2O$	0.1
Iron Source	
(Na_2)EDTA	20
$FeSO_4.7H_2O$	15
Vitamins	
Nicotinic Acid	5.0
Thiamine HCl	5.0
Pyridoxine HCl	0.5
Others	
myo inositol	1000
Sucrose	30000
2,4-D	0.5
Kinetin	0.1

2.2.1.2 Germination medium

Sterile seeds were germinated on 20ml. of medium which consisted of 6g. agar dissolved in a litre of distilled water.

2.2.1.3 Preparation of growth regulator stock solutions

The following growth regulators were used to prepare stock solutions for use in the different media. All growth regulators were obtained from Sigma Chemical Co.

Kinetin (6-Furfurylaminopurine)

Naphthalene Acetic Acid (NAA)

2,4-Dichlorophenoxyacetic acid (2,4-D)

6-Benzylaminopurine (6-BAP)

Zeatin (6-(4-Hydroxy-3-methylbut-2-enylamino)purine)

Indole-3-Acetic Acid (IAA)

Stock solutions were prepared by dissolving a known amount of each substance in 5ml. 0.1M. KOH, then slowly adding distilled water to achieve the required concentration in a volumetric flask. These stock solutions were stored at 4°C. and were routinely replaced every 4 weeks. The heat labile growth regulators, IAA and NAA, were filter sterilized and pH adjusted, with 10N. HCL, then added to the medium after autoclaving. The filter sterilization procedure is described in Section 2.2.2.2.

2.2.2 Sterilization techniques

It is vital that all types of infection should be avoided when working with tissue culture. This means that all glassware, media and plant material was sterilized prior to use. The sterilization procedures used were as follows;

2.2.2.1 Sterilization by heat

All glassware, distilled water and nutrient media which did not contain heat labile substances were sterilized by autoclaving at 121°C. for 20min. at a steam pressure of 15 psi.

2.2.2.2 Sterilization by filtration

The sterilization of heat labile substances eg: IAA, NAA was performed using preautoclaved Millipore Swinnex-13 filtration units containing a Millipore cellulose acetate filter (0.22μM pore size) (Millipore, Molsheim, France). Liquids were passed through these units into a sterile environment ie: media or sterile beaker, using a sterile plastic syringe (Becton Dickinson & Co, Ireland) the size of which depended on the volume of liquid to be filtered.

2.2.2.3 Sterilization with hypochlorite

All seed and explant material was sterilized in sodium hypochlorite prior to their inoculation onto the medium.

a. Sterilization of seeds

Seeds, contained in a muslin bag, were surfaced sterilized by a rapid pre-sterilization in 95% (v/v) ethanol (EtOH) for 30sec. followed by immersion in 10% (v/v) sodium hypochlorite, containing 1.5% available chlorine (BDH), for 30min. Seeds were then removed from the sterilant under sterile conditions and washed three times in sterile distilled water prior to their inoculation.

b. Sterilization of stem and leaf explants

All plant material was sterilized after excision from the plant and prior to inoculation onto the medium. Stem internode and leaf material was taken from the upper (sub-apical) or lower regions of healthy, actively growing plants. All cut ends were sealed with wax (Paraplast-Lancer, Eire) and allowed to dry prior to sterilization. After a rapid pre-sterilization in 70% (v/v) EtOH for 30sec. explants were exposed to 10% (v/v) sodium hypochlorite for 30min. then washed three times in sterile distilled water under sterile conditions prior to inoculation.

2.2.2.4 Sterile inoculation and transfer technique

All cell culture manipulations were carried out in a laminar air-flow cabinet (design as Flow labs., Irvine, Scotland). All surfaces were swabbed with EtOH prior to use. All instruments used in manipulations ie: forceps, spoon spatula, cork borer were stored in EtOH and flamed before use.

2.2.3 Initiation and maintenance of cultures

2.2.3.1 Standard culture conditions

Unless otherwise stated in the text *in vitro* plant material was grown in a culture room under the following cultural conditions.

TEMPERATURE - 25°C. +/-1°C.

PHOTOFLUX DENSITY/ILLUMINANCE - 25 $mol.m^{-2}. sec.^{-1}$ 1050 lux

LIGHT SOURCE - Compton Warmwhite fluorescent

LIQUID CULTURE AGITATION - Continuous rotation in a horizontal plane, 98rpm., 0.8cm. amplitude.

2.2.3.2 General procedure for the initiation and maintenance of cultures

a. Callus cultures

After sterilization and washing, the plant material was transferred to a sterile dish in the laminar air flow cabinet. Transverse segments of stem internode were made using scalpel and forceps. Leaf explants were separated into petiole and lamina, the petiole was cut into segments like the stem, while from the lamina discs were cut out from either side of the midrib using a flamed cork borer. These segments and discs were placed on 25ml. SH medium contained in sterile polystyrene Petri dishes (Sterilin) which were then covered and sealed with a single layer of parafilm (American Can Company). These plates were then left on shelves under the standard culture conditions. Callus growth was rapid enough to allow it to be isolated from the explant after 3-4 weeks when it was subcultured to fresh medium, afterwhich a subculture was made every four weeks.

b. Suspension cultures

Suspension cultures were initiated by adding 2-8g. of friable callus to 50ml. liquid medium contained in 250ml. conical (Erlenmeyer) flasks which were covered with a double layer of aluminium foil. These flasks were placed on a rotary shaker under the standard culture conditions and subculture of tissue using a perforated spoon spatula was carried out every 4 weeks.

c. Immobilized cultures

Suspension cultures were immobilized in $1cm^3$ polyurethane foam blocks (Declon, Corby, UK) using the procedure described by Lindsey *et al.* (1983). Foam blocks were boiled for 1 h. in distilled water then washed in EtOH to remove any toxic substances. After drying, six blocks were placed into a 50ml. plastic centrifuge tube (Corning) and then sterilized by autoclaving. Under sterile conditions the foam blocks were dropped into a flask containing a newly subcultured, friable cell suspension. After four weeks on a rotary shaker the cells invade the pores of the foam blocks and become immobilized in them.

2.2.3.3 Germination of sterile seeds

Seeds washed of all sterilant were transferred to the germination medium contained in Petri dishes and *ca.* 12 seeds were spread evenly over the surface of the media. The Petri dishes were sealed with parafilm and left in the standard growth conditions for up to six weeks or until they had grown sufficiently to allow the dissection of hypocotyls from the intact seedlings which were used in the regeneration experiment.

2.2.3.4 Procedure for the isolation of single cells

A cell plating technique was used to isolate single cells from a friable suspension culture. Single cells were sieved through a series of nylon mesh filters down to 100μM in size, then centrifuged at 2000xg for 5min. and diluted with SH medium to achieve a volume of approximately 50,000 cells ml^{-1}. The cells were then mixed with an equal volume of SH medium with 1% low temperature agarose (Sigma Chemical Co.), to produce a dilution of 25,000 cells/ml., and then plated out on a gridded Petri dish. The plates were examined microscopically for single cells which were marked so that their growth could be followed. When the colonies were approximately 1mm. in diameter they were pricked out and transferred to SH medium where they developed into callus cultures which were then subcultured every four weeks under the standard culture conditions.

2.3 Analytical techniques

2.3.1 Analysis of morphological characteristics

2.3.1.1 Determination of the initial and final fresh weight

The initial fresh weight was determined by weighing the Petri dish or flask containing the new medium before and after the cells had been transferred at subculture and calculating the difference between these two weights. The final fresh weight was determined by adding the weight of the cells that had not been transferred to the initial subculture weight. Callus and immobilized cells were removed from the old medium with forceps while suspension cultures were removed with a perforated spoon spatula. After the weight had been taken the cells were filtered through Whatman no. 2 paper prior to the analysis of dry weight, appearance, RGR, TCC and TPC, while the medium was filtered using the same procedure before the analysis of the SPCs.

2.3.1.2 Determination of dry weight

A known weight of cells was placed in a pre-weighed paper cup and dried overnight in a 60°C. oven, they were then cooled in a desiccator and weighed.

2.3.1.3 Determination of appearance

The appearance of callus, suspension and immobilized cultures was determined at each subculture with a quantitative assessment of both colour and texture using the following descriptive terms.

TEXTURE	COLOUR
Friable (F)	Yellow (Y)
Fine (Fn)	Green (G)
Aggregated (A)	White (W)
Immobilized (I)	Brown (B)

2.3.1.4 Determination of relative growth rate

The relative growth rate (RGR) was determined by subtracting the log value of the initial wet weight (W_2) from the final wet weight (W_1) of the cells from callus, suspension or immobilized regimes, and dividing this value by the length of time (T_2-T_1) in days that the cells were in culture, as follows;

$$\frac{\text{Log } W_2 - \text{Log } W_1}{T_2 - T_1} = \text{RGR}$$

The results were expressed in $day^{-1} \times 10^{-2}$

2.3.1.5 Determination of the stage and type of regeneration using an arbitrary scale based on morphological characteristics

An arbitrary scale was prepared in order to provide a qualitative measure of callus regeneration. This involved scoring the growth, colour, shoot and root formation using a numbering system from 1-5. The scoring system is shown in Table 3.4.2.

2.3.2 Chemical analysis of cultures

2.3.2.1 Extraction and determination of total chlorophyll content

The extraction and determination of the total chlorophyll content (TCC) from cells was carried out using the method of Harborne (1976). A known weight of filtered cells was ground on ice for 1min. in 5ml. of cold 80% (v/v) acetone (BDH) using a pestle and mortar (immobilized blocks were cut up with a scalpel prior to grinding). The procedure was then repeated with another 4ml. 80% acetone after which the total mixture was filtered through Whatman no.1 into a 10ml. volumetric flask. The filtrate was then made up to the 10ml. mark using 80% acetone. The optical density (OD) of the filtrate was determined spectrophotometrically at 663 and 645nm on a Pye-Unicam S18-100 spectrophotometer using 80% acetone as the reference blank. The total chlorophyll concentration in the extract was estimated using the following formula;

$$TCC = 20.2 \times OD_{645} + 8.02 \times OD_{663}$$

Results expressed in $\mu g\ g\ DW^{-1}$ of cells.

2.3.2.2 Extraction and determination of total protein

a. Extraction of total protein

Total protein was taken up in a mild alkali solution by grinding cells (*ca.* 1g) in 5ml. of 0.1M. NaOH using a pestle and mortar (immobilized blocks were cut up with a scalpel prior to grinding). This procedure was repeated with a further 5ml. of alkali after which the extract (cells and alkali) were pooled into a 15ml. screw top centrifuge tube and left overnight at 4°C. This preparation was then centrifuged at 2000xg for 10min. and the supernatant poured off into another centrifuge tube. A small volume of this extract (*ca.* 1ml) was pipetted off into a 1.5ml. Epindorff tube and centrifuged for 5min. in a microcentrifuge at 12,000xg to remove any remaining debris, after which the sample was ready to assay.

b. Determination of total protein

The procedure for the determination of the total protein content (TPC) of cells was adapted from the method of Bradford (1976) which describes the quantitation of microgram quantities of protein using a protein-dye binding assay.

b. i. Preparation of dye reagent, acetate buffer and standard solutions

200 mg of Coomasie Brilliant Blue G-250 (Sigma Chemical Co.) was dissolved in 200ml. of 85% (v/v) phosphoric acid (BDH) to give a final concentration of 1 mg ml^{-1}. This was then diluted 5-fold with distilled water, filtered through Whatman No.2 to remove any undissolved solids and stored at room temperature. The reagent was replaced every six months.

A volume of 250ml. of 0.5M. sodium acetate ($CH_2COO.Na_2$) was added to 250ml. of 0.5M. glacial acetic acid (CH_2COOH) to give a 0.25M. acetate buffer solution with a pH of 4.5. This was also stored at room temperature.

Bovine Serum Albumin (BSA, Sigma Chemical Co.) was used as the protein standard. A range of standard solutions made up to different concentrations (from 50µg ml^{-1} to 200µg ml^{-1}) using 0.1M. NaOH, were prepared immediately before use from a 200µg ml^{-1}(w/v) BSA stock solution.

b. ii. Assay procedure

A 0.2ml. aliquot of sample extract, standards and blank (0.1N NaOH) were added to clean glass test tubes. To each test tube 3.8ml. of acetate buffer and 4.0ml. Coomassie Blue Reagent were added using a 5ml. glass pipette. The solutions were thoroughly mixed for 1 min. and then allowed to stand for a further 5min. to enable the dye-protein complex to form. The optical density of the mixture was determined spectrophotometrically, in glass cuvettes, at 595nm. using 0.1M. NaOH as the reference blank. A typical calibration curve using the BSA standards is shown in Fig. 2.3.2 i. and from this the total protein content was calculated and finally expressed as mg g DW^{-1} of cells.

2.3.2.3 Extraction and determination of phenylalanine ammonia lyase activity

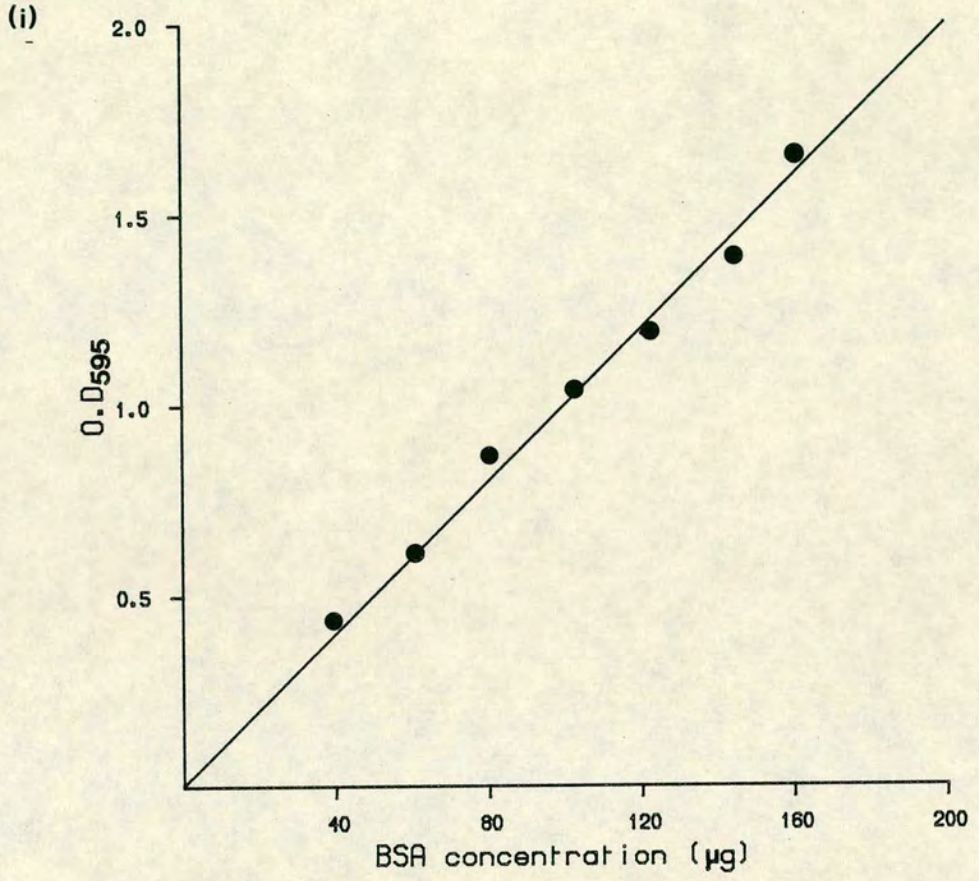
The procedure for the extraction of the phenylalanine ammonia lyase (PAL) enzyme was adapted from a method described by Ozeki and Komamine (1985). The assay and spectrophotometric determination of enzyme activity was taken from Tanaka *et al.* (1974) most of which was a modification of the method described by Zucker (1965). The specific activity of the enzyme is expressed as the amount of substrate, in the form of exogenously supplied phenylalanine, that is converted to cinnamate, the product, per unit of total protein in the enzyme extract.

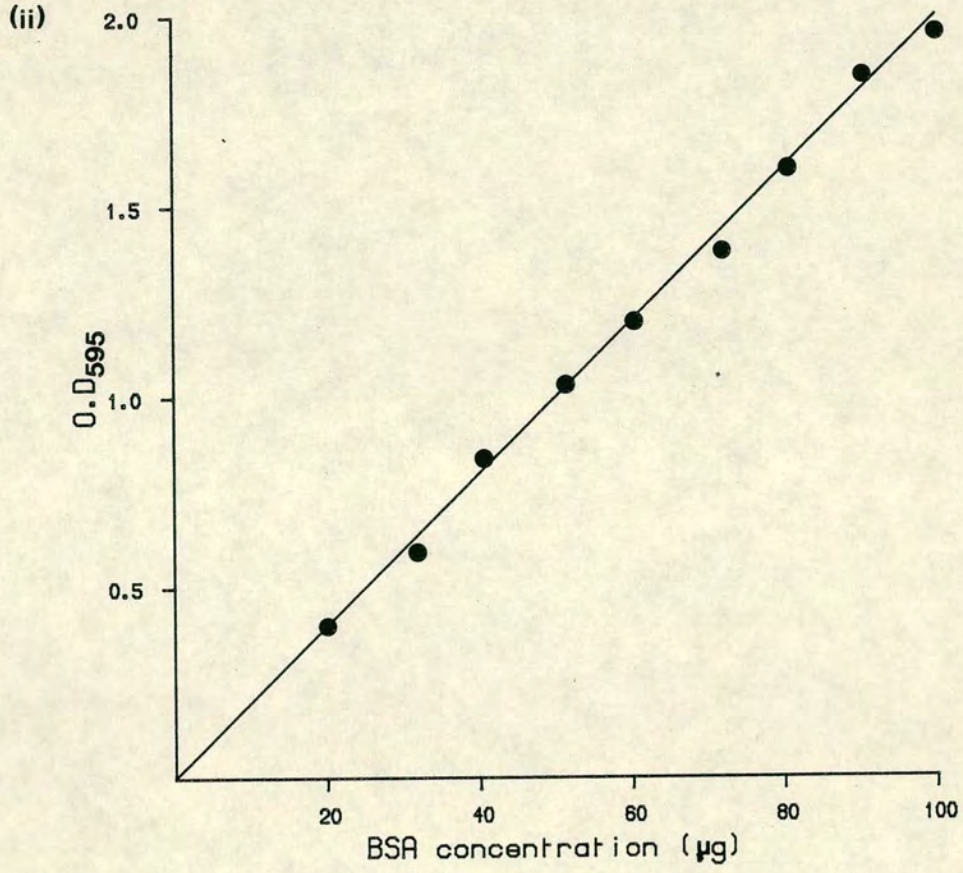
a. Buffer and substrate preparation

Two TRIS-HCl buffer solutions (pH 7.8, 200mM) were required for the extraction of the enzyme; one with and one without the addition of 10mM 2-mercaptoethanol (Sigma Chemical Co.). The buffer was prepared by the addition of 25ml. 0.2M. TRIS (Trisma Base, Sigma Chemical Co.) to 35 ml. 0.1M. Hydrochloric acid (HCl, BDH) which was then made up to 100 ml. with distilled water. When required the 2-mercaptoethanol was added to 100 ml. of buffer. Both buffers were stored at 4°C. A 50mM solution of L-phenylalanine (Sigma Chemical Co.), the enzyme substrate, was prepared by dissolving 0.826g in 100ml. buffer (without mercaptoethanol) which was stored at 4°C.

Figure 2.3.2

Calibration curves prepared using BSA for the calculation of (i). total cellular protein and (ii). enzymatic protein





b. Enzyme extraction

Cells (*ca* 2g.) were homogenised with 0.2g. Polyclar AT (BDH) in 3ml. buffer (with mercaptoethanol) on ice for 1min. using a pestle and mortar. The homogenate was then poured into a 15ml. centrifuge tube and the remaining cells in the mortar were washed out with a further 3ml. of buffer and added to the initial homogenate. The cell debris was removed by centrifugation at 2,000xg for 20min. after which the total supernatant was poured into another 15ml. centrifuge tube containing 0.2g Dowex 1*4 (Sigma Chemical Co.) with 2ml. buffer (with mercaptoethanol). This was mixed thoroughly and placed on ice for 20min. Finally the extract was spun down at 2,000xg for a further 20min. The supernatant was carefully poured into a mixing tube and mixed well before being used in the assay.

c. Determination of total protein in enzyme extract

Protein was extracted by precipitation by adding 0.1ml. 100% (v/v) trichloroacetic acid (TCA) to the protein extract and leaving the mixture overnight. The precipitate was centrifuged down at 5,000xg for 10min., and the supernatant poured off and the pellet redissolved in 1ml. 0.1M NaOH. Protein was then determined using the micro-protein assay described by Bradford (1976). Using a 100µg l^{-1} (w/v) stock solution of BSA a range of standard solutions was made up to different concentrations (20µg l^{-1} to 100µg l^{-1}) prior to use with 0.1M NaOH. A typical calibration curve for the standards is given in Fig 2.3.2 ii.

d. Assay and determination of enzyme activity

The optical density was determined as soon as the substrate was added to the extract and after a 2h. incubation period. Test tubes containing 3ml. buffer (without mercaptoethanol) for the assays, and one containing 4ml. buffer for the blank, were set up in a 40°C. water bath. To each of the three tubes 0.3 ml. enzyme extract was added. After 10min. enough time to allow the mixtures to equilibrate, the 2h. assay tube received 1ml. of the substrate solution. A 1ml. of substrate was then added to the other assay tube and the optical density of this mixture was determined spectrophotometrically at 270nm. using the blank (without substrate) as the reference. This procedure was then repeated after 2h. with the remaining assay tube using the same blank for calibration.

The difference in optical density (O.D) found over the two hour period and the total protein content (TP) of the extract was used to calculate the specific activity of the PAL enzyme using the following equation;

$$\frac{4.3 \times O.D._{diff} \times 10^{15}}{1.497 \times 10^{11} \times \mu g\ TP}$$

The results are expressed in $\mu kat\ kg^{-1}$ protein

2.3.2.4 Extraction and determination of soluble phenolic compounds

The following procedures have been developed from the work of Hall *et al.* (1987) and Lindsey (1982) and describe the extraction and determination of the soluble phenolic compounds (SPCs) in the medium. Capsaicin, related capsaicin intermediates and unknown phenolics are found in these fractions.

a. Extraction of total phenolics

a. i. From liquid culture medium

The liquid medium of suspension and immobilized cultures was extracted three times in 50ml. chloroform ($CHCl_3$, BDH) using a separating funnel. Each time the $CHCL_3$ extract was taken off and pooled in a flask containing *ca.* 5g. sodium sulphate (Na_2SO_4, BDH) which was used to remove any excess water. The pooled extract was then evaporated to dryness *in vacuo* at 60°C. and the residue was taken up in 1ml. methanol (HPLC grade, Fisons). The extracts were then filtered using a microfilter fitted with a 0.45µl pore filter (Scotlab) and stored in crimped capped HPLC glass vials at 4°C.

a. ii. From agar culture medium

The agar medium of callus cultures was extracted by mashing in 20 ml. $CHCL_3$ using a pestle and mortar. The solvent was poured into a 250 ml. conical flask and the remaining medium remashed with another 20ml. $CHCL_3$. The total homogenate was then added to the first filtrate with a further 110ml. $CHCL_3$ and mixed for 1h., after which the $CHCL_3$ was filtered off, dried down and stored as described in Section 2.3.2.4.a i.

b. Determination of soluble phenolic content by High Performance Liquid Chromatography (HPLC)

b. i. Preparation of solvents

The solvents used (methanol, water and acetic acid) were all HPLC grade (Fisons Ltd, Loughborough, U.K). They were filtered at reduced pressure through 0.45µm pore Nylon-66 membrane filters (Scotlab, Glasgow) using a Millipore microfilter system. The solvents were degassed with helium gas immediately before use.

b. ii. Preparation of standards

A mixture containing six authentic standards was prepared for HPLC analysis they were obtained from Sigma Chemical Co., Poole, UK or Aldrich Chemical Co., Gillingham, UK and these were run in conjunction with the extracts at the beginning and end of each analysis day. All standards used are listed below and were dissolved to a concentration of 1mg l^{-1} in methanol. From each standard a 1ml. aliquot was taken and pooled from which 1ml. was taken and filtered and then stored in vials. Calibration curves for each standard were prepared from which the relative amounts of each known capsaicin related compound found in the medium could be determined (Fig 2.3.3).

Caffeic acid (3,4-dihydroxycinnamic acid)

Vanillin (4-hydroxy-3-methoxybenzoic acid)

Coumaric acid (4-hydroxycinnamic acid)

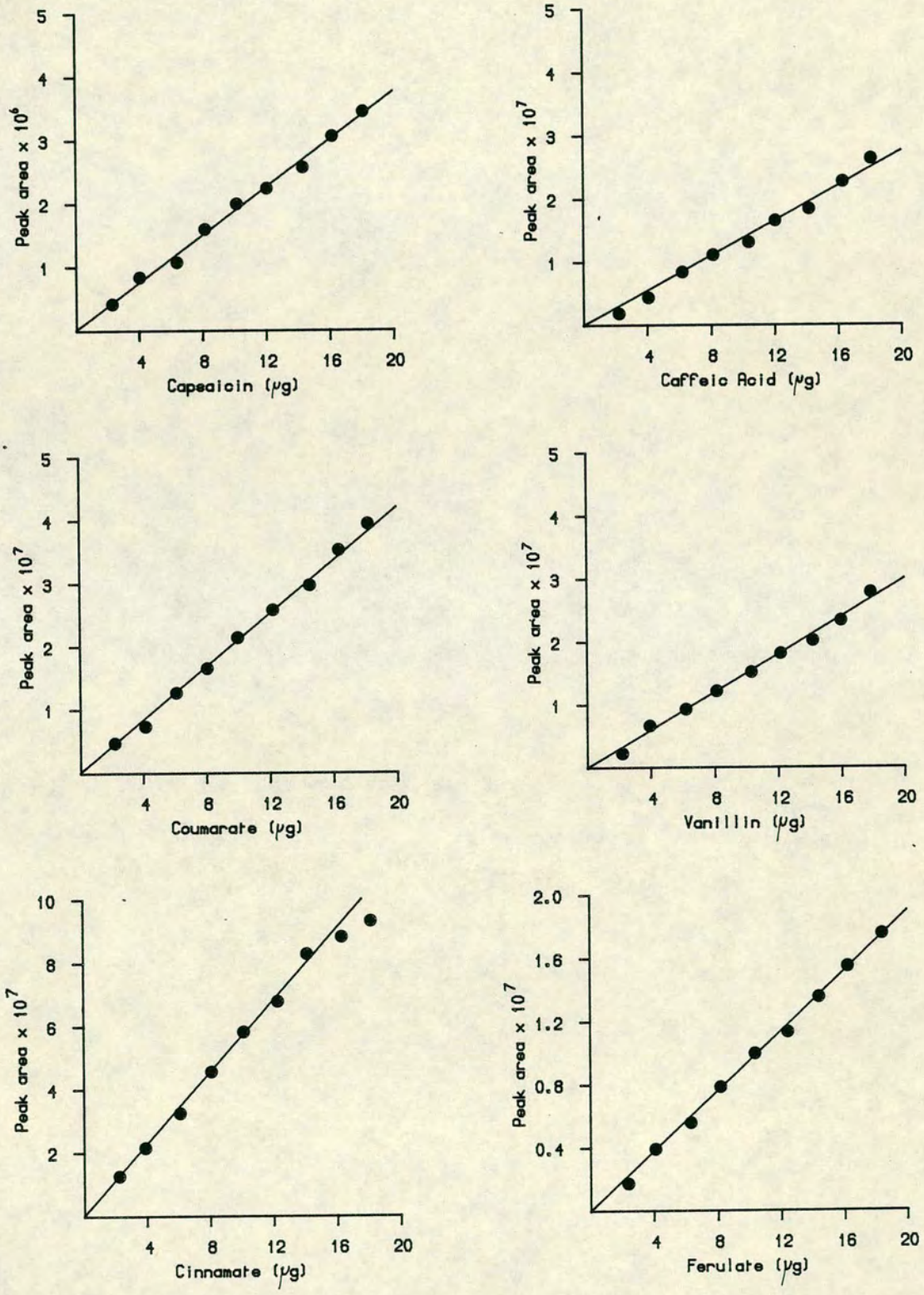
Ferulic acid (4-hydroxy-3-methoxycinnamic acid)

Cinnamic acid (β-phenylacrylic acid)

Capsaicin (8-methyl-N-vanillylnonanamide)

Figure 2.3.3

Calibration curves of authentic capsaicin-related standard compounds



b. iii. Analysis of samples

10µl sample extracts were injected into a Hewlett-Packard HP1090 liquid chromatograph using a binary DR5 solvent delivery system and autosampler (Hewlett-Packard, Reading, UK). Capsaicin, known and unknown SPCs were separated over a 30 min. run as peaks, using a 200*5 mm., 5µm Hypersil MOS (C8) column (Phase Sep, Clwyd, UK) at 40°C with a varying mixture of methanol and 5% acetic acid in water as the eluting solvents. The chromatograph was used in conjunction with an HP1040 Diode Array Detector (DAD) system (Hewlett-Packard) which identified each phenolic peak by its characteristic spectrum between 240nm and 352nm. The names and characteristic spectra of all unknown SPCs found in the medium of the cultures are shown in Fig 2.3.4. The area and percentage area of peaks of all known SPCs were determined after every run. A typical chromatograph showing these standards and their characteristic spectra is shown in Fig 2.3.5.

2.3.2.5 Statistical Analysis

The main statistical analysis used in this investigation was a multifactor analysis of variance (ANOVA) to determine the significance of differences among treatment means of more than one factor (Snedecor and Cochran 1967). The level of significance is determined from the variance ratio (VR) which was found using a GENSTAT (ANOVA) statistical package obtained from the computing facilities of the Edinburgh Regional Computing Centre (Talbot 1981, Alvey *et al.* 1982).

To determine the significantly different means a Student's range was used from which the least significant difference (LSD) was obtained to compare among means (Snedecor and Cochran 1967). To compare the significance of the difference between two means a two sample t-test was used and the VR from this and all other tests were checked at the p=0.01 level of significance using the tables found in Parker (1979).

Figure 2.3.4

Spectra (240–352nm.) of unknown $CHCL_3$ soluble phenolic compounds found in the medium of callus, suspension and immobilized cultures.

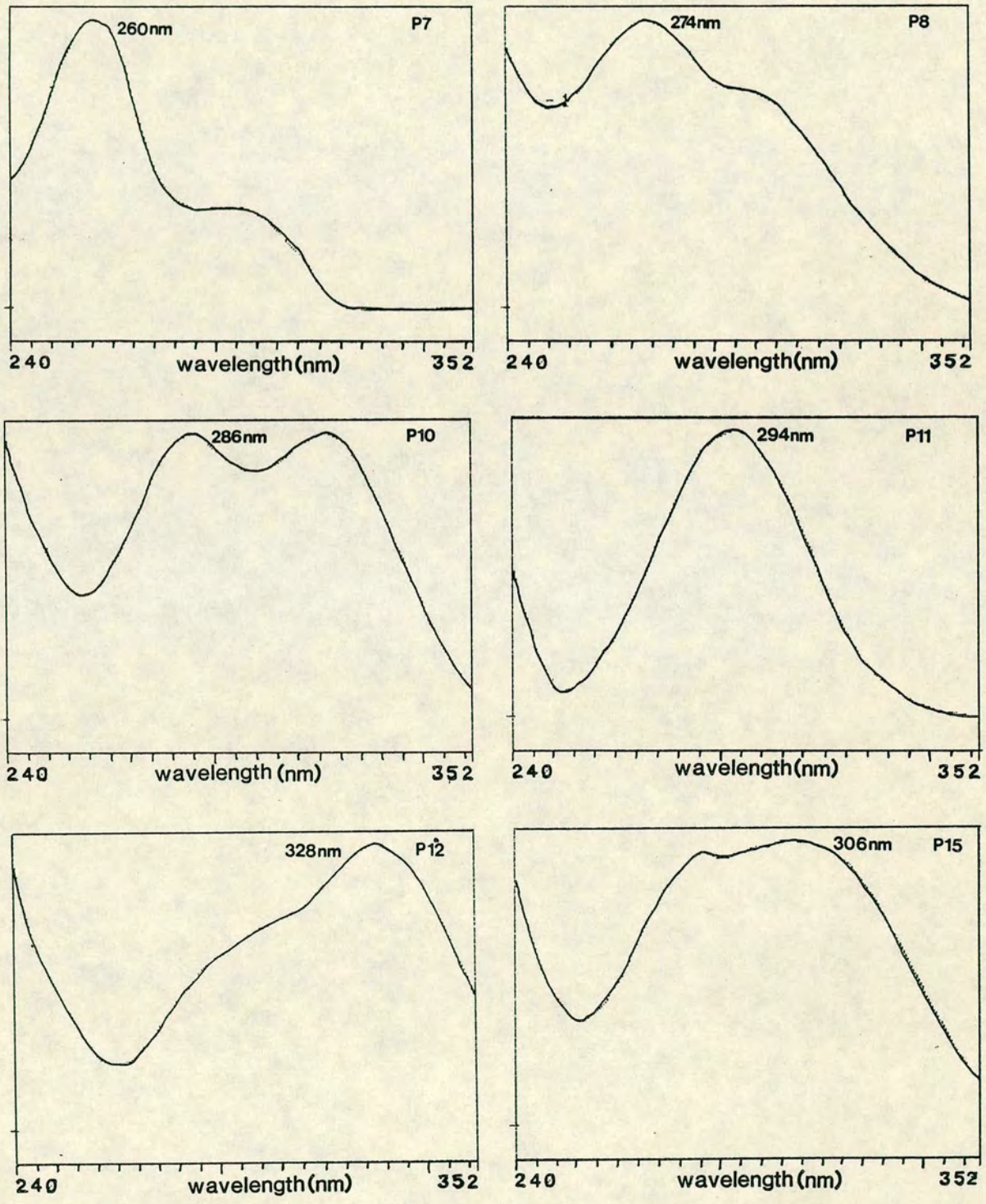
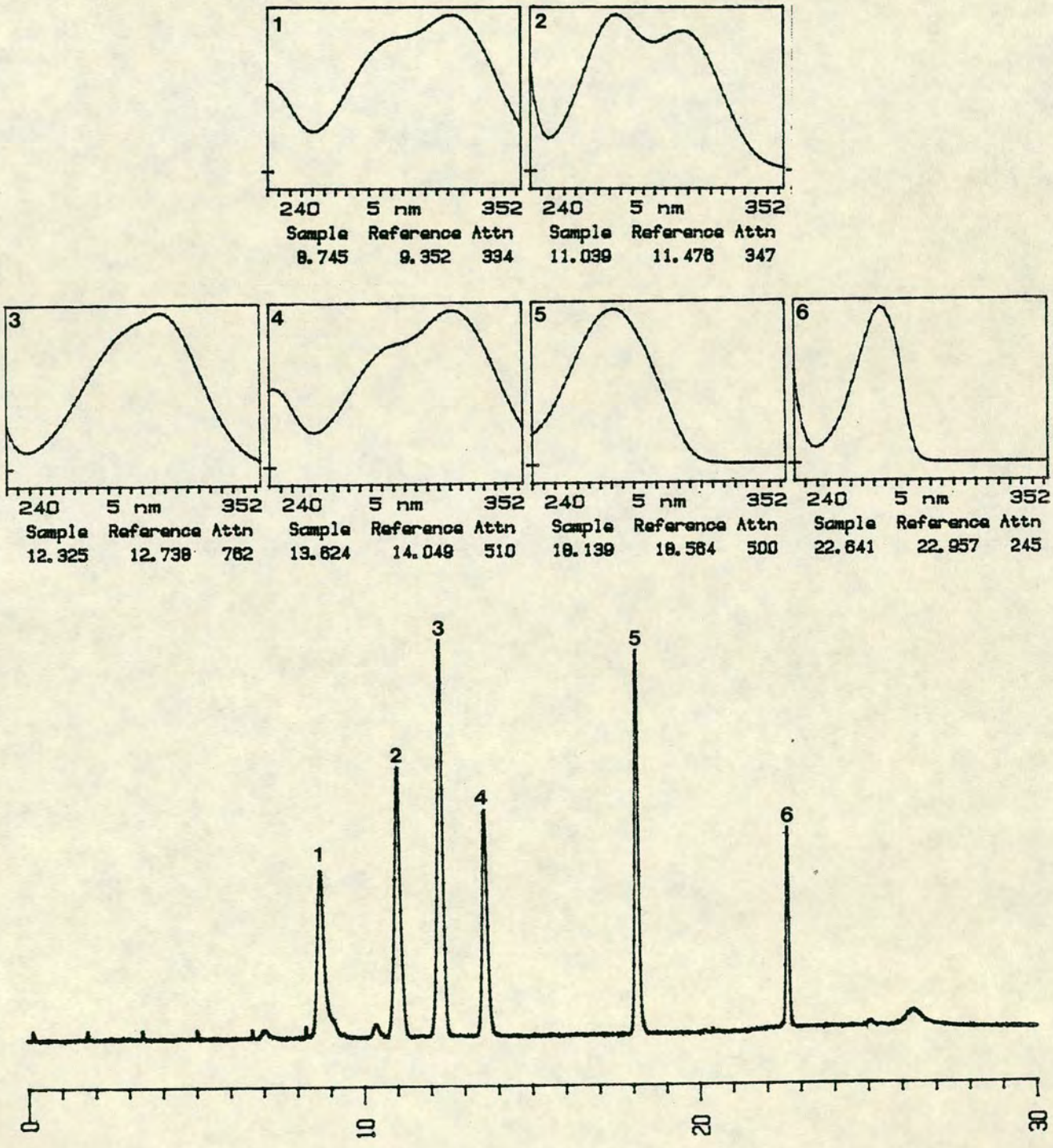


Figure 2.3.5

A chromatogram of a standard solution of capsaicin and related phenolic compounds and the times they appear over a 30min. run. The spectra (240-352nm.) of the eluted compounds are drawn above. The compounds were as follows; 1. Caffeic acid, 2. Vanillin, 3. Coumaric acid, 4. Ferulic acid, 5. Cinnamic acid and 6. Capsaicin.



CHAPTER THREE

RESULTS

PART ONE

COMPARISONS AMONG AND WITHIN CLONES ISOLATED FROM A SINGLE SUSPENSION CULTURE

3.1 Comparisons among and within clones isolated from a single suspension culture

It is now well established that genetical, morphological and biochemical differences arise among and within cultures derived from single cells or protoplasts isolated from a cell population (Dougall 1985). The following investigation consists of a series of experiments which were carried out to determine the nature and degree of variability that arises first among clones and second within a single clone maintained under the same or different culture regimes over a series of successive subculturing.

3.1.1 Comparison of morphological and metabolic characteristics among clones

The first part of the investigation involved two experiments and was carried out to determine whether there were any differences in the morphological and metabolic characteristics among clones which had been established in this laboratory eighteen months previously. This involved, firstly a comparison of twenty callus clones over a four week period, followed by a more detailed examination of five of the clones maintained as callus or in suspension over a sixteen week period with a subculture every four weeks.

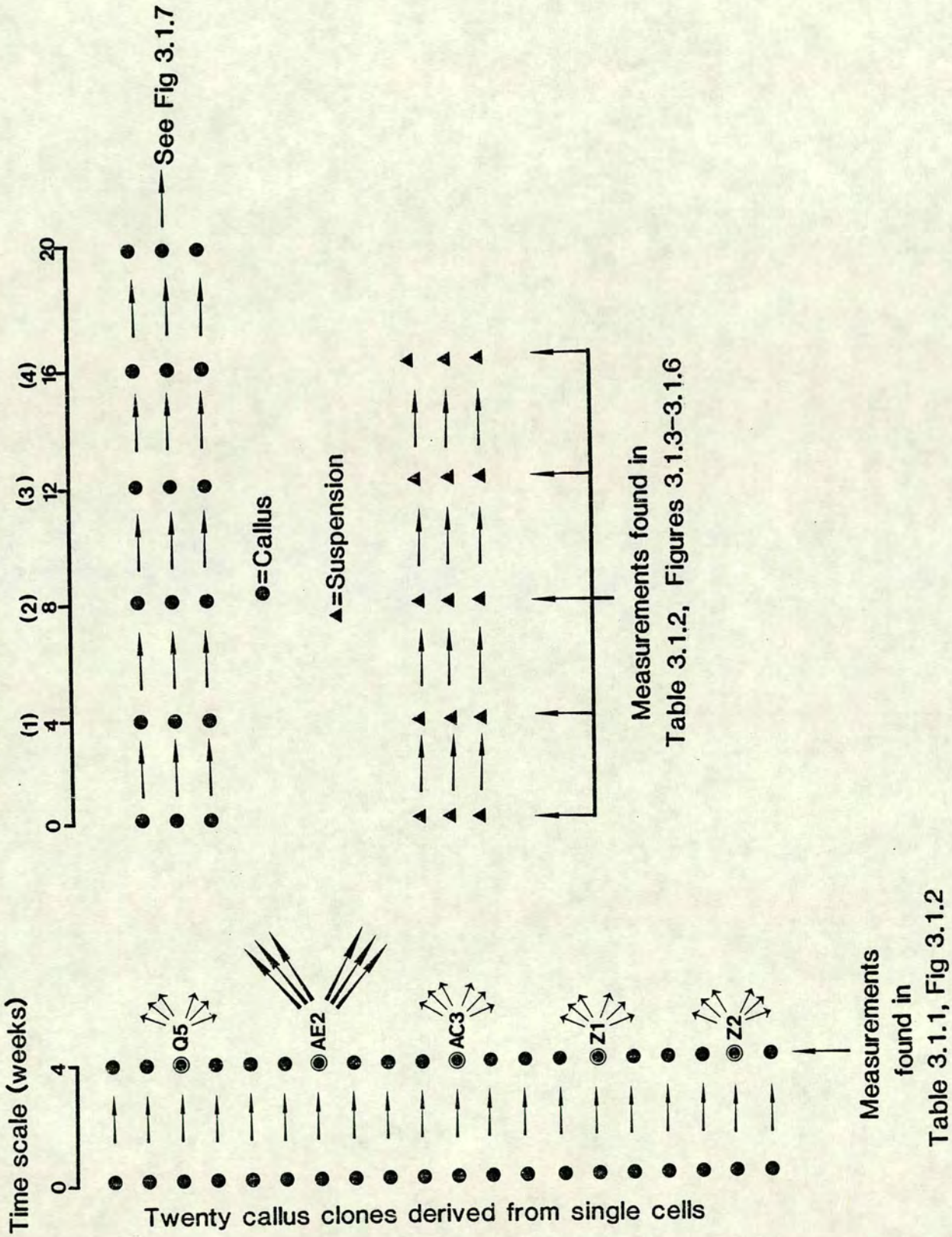
3.1.1.1 Experimental procedure (see Fig. 3.1.1)

Single cells had been isolated from an apparently homogeneous suspension culture of *Capsicum frutescens* cv. Cayenne using the procedure described in Section 2.2.3.4. From these cells two hundred callus clones were established and maintained under standard culture conditions with a subculture every four weeks.

After eighteen months and at the beginning of the current work around one hundred callus clones remained from the original number and from this population twenty clones were selected at random. This current work began when these twenty clones were subcultured and maintained for four weeks after which the appearance, relative growth rate (RGR) and total chlorophyll content (TCC) were determined.

Figure 3.1.1

Diagram showing the origins and relationships of experimental material and the points in the first twenty weeks when data were collected.



Five clones showing different morphological and metabolic characteristics were then selected out for further study. Each one of these five clones was randomised from which six cultures were obtained by subculture, three of which were maintained as callus and three as suspensions providing replication for the analysis of variance. The six cultures of each clone were maintained in this way for sixteen weeks during which one subculture of each was made every four weeks. The appearance and RGR of the cells and the soluble phenolic compounds (SPCs) in the medium were determined using the material remaining after each subculture.

Using these characteristics, comparisons of average or mean values were made among the five clones in both regimes and between the two culture regimes over the sixteen week subculture period. An analysis of variance was used to calculate the variance ratios and LSD values to determine any significant differences in the RGR that emerged among clones and between regimes during the experiment.

3.1.1.2 Comparison among twenty callus clones over four weeks

There were considerable differences in the appearance, RGR and TCC among the twenty callus clones after four weeks (Table 3.1.1). Both the friability and colour varied considerably among these cultures from brown and aggregated in some, eg: clone N4, to yellow, green and friable in others, eg: clone AE2 (Table 3.1.1, Fig 3.1.2). It was also noticeable that the RGR of these callus clones differed appreciably, from those which were slow growing, in the case of the clone BV7, to those which were fast growing, as in clone AC2 (Table 3.1.1). The slow growing clones were nearly always aggregated, while the fast growing clones were friable (Table 3.1.1, Fig 3.1.2). White and green calluses were also apparent, the green cultures having quantifiable amounts of chlorophyll, while other callus clones were brown (Table 3.1.1, Fig 3.1.2). The clones AE2 and AC3 which had noticeably higher RGR values with a yellow or green and friable appearance were selected, with three others which had contrasting morphological patterns (clones Z1, Z2 and Q5), for a more detailed analysis of differences among clones (Table 3.1.1, Fig 3.1.2).

Table 3.1.1

Appearance, relative growth rate (RGR) ($day^{-1}x10^{-2}$) and total chlorophyll content (TCC) ($\mu g\ DW^{-1}$) of twenty selected callus clones over a four week period.

	Clone	Appearance	RGR	TCC
	BZ4	Y(W),A	0.78	N.D
	AG2	Y(G),F	1.83	10.6
	CD8	Y(W),F(A)	1.23	N.D
	N4	B,A	0.94	N.D
	AC2	Y(V),F	2.73	4.5
*	Q5	Y(G),F(A)	1.48	7.8
	Y3	G(Y),F	0.73	9.2
	BZ4	Y(B),A	0.96	N.D
*	AE2	Y(G),F	4.28	4.2
	CD5	W(V),A(F)	2.00	N.D
	EF4	Y(B),A	1.76	N.D
	AC1	Y(B),A	0.12	N.D
	BV7	Y,A	0.74	N.D
	CB6	W(B),F	1.94	N.D
	N3	Y(G),F(A)	1.11	14.2
	AB9	Y(V),F	0.54	N.D
*	AC3	Y,F	2.76	N.D
*	Z1	Y(V),F(A)	2.59	6.6
*	Z2	Y,F	2.12	N.D
	BQ3	Y,A	0.78	N.D

* Clones selected out for further analysis

N.D = Not detectable

Y=Yellow

G=Green

B=Brown

W=White

F=Friable

A=Aggregated

V=Variegated

Y(G) means culture mainly yellow with the presence of a small amount of cells of one other colour, in this case green.

Y(V) means culture mainly yellow with the presence of small amounts of other coloured cells (in this case white, green or brown) giving the culture a variegated appearance.

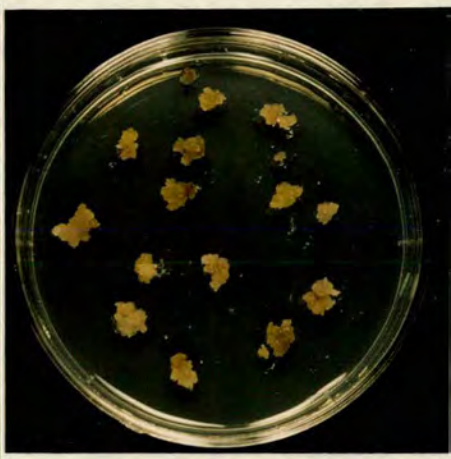
F(A) means culture mostly friable with small amounts of aggregated cells.

Figure 3.1.2

The appearance of nine callus clones after two weeks of the four week subculture period.

AE2

AC3



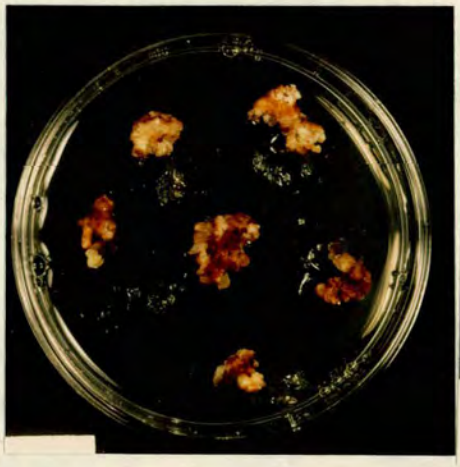
Q5

BZ4

Y3

Z1

Z2



N4

AG2

3.1.1.3 Comparison among the five clones within callus and suspension regimes over sixteen weeks

a. Appearance of the clones

The appearance of the five selected clones maintained as callus or in suspension changed appreciably over the sixteen weeks (Table 3.1.2). Clone AE2 was notable for the stability of its yellow and friable appearance while the other four clones showed a progressive change in appearance; from friable to aggregated, and more markedly, from yellow to brown with increasing subculture (Table 3.1.2). These changes were more obvious in suspension cultures (Table 3.1.2, Fig 3.1.3). The clones AC3, Z1, Z2 and Q5 all exhibited similar qualitative variation but differed in the rates of change, in particular AC3 was slow to change while clone Q5 changed quickly and had developed the brown and aggregated growth pattern by the second subculture (Table 3.1.2, Fig 3.1.3).

b. Relative growth rate (RGR) of the clones

It can be seen from the results of the analysis of variance in Table 1 of the Appendix that the variance ratios for clones and subcultures are both significant, which shows that there were significant differences in the RGR among clones and that the RGR of the clones changed significantly with subculture.

The results presented in Fig 3.1.4. a. and b. show that the mean RGR of clone AE2 was significantly greater than the other four clones, a result that although apparent in both regimes was more significant in suspension culture. The RGR of clone AC3 was significantly greater than the remaining three clones, a result that was attributed to the similar values in both regimes (Fig 3.1.4. a, b). The RGR of clones Z1 and Z2 were not significantly different from each other in either regime, but due to the RGR of the callus cultures their RGR values were significantly greater than that of clone Q5 which had low RGR values in both callus and suspension regimes (Fig 3.1.4. a, b). The results presented in Fig 3.1.4. c and d show that there was a significant and consistent drop in the RGR with increasing subculture, a pattern apparent in all five callus and suspended clones.

Table 3.1.2

Appearance of five selected clones maintained as callus (C) or in suspension (S) over sixteen weeks with a subculture and measurement every four weeks. Each score is an average of three sets of observations.

		Appearance			
Clones	**Parent**	**Week 4**	**Week 8**	**Week 12**	**Week 16**
AE2 C	Y(G),F	Y,F	Y,F	Y,F	Y,F
AE2 S	Y(G),F	Y,F	Y(B),F	Y,F	Y,F
AC3 C	Y,F	Y,F	Y(B),F	B(Y),F	B(Y),F
AC3 S	Y,F	Y(B),F	B,F(A)	B,A(F)	B,A(F)
Z1 C	Y(V),F(A)	Y(B),F(A)	Y(B),F(A)	B(Y),F(A)	B(Y),F(A)
Z1 S	Y(V),F(A)	B,F(A)	B,F(A)	B,A	B,A
Z2 C	Y,F	Y(B),F	Y(B),F	Y(B),F	B(Y),F
Z2 S	Y,F	B,F(A)	B,F(A)	B,A	B,A
Q5 C	Y(G),F(A)	Y(G),F(A)	B,F(A)	B,F(A)	B,F(A)
Q5 S	Y(G),F(A)	B,F(A)	B,A	B,A	B,A

Y=Yellow

G=Green

B=Brown

W=White

F=Friable

A=Aggregated

V=Variegated

Y(G) means culture mainly yellow with the presence of a small amount of cells of one other colour, in this case green.

Y(V) means culture mainly yellow with the presence of small amounts of other coloured cells (in this case white, green or brown) giving the culture a variegated appearance.

F(A) means culture mostly friable with small amounts of aggregated cells.

Figure 3.1.3

The appearance of five clones in suspension after the last subculture of a sixteen week period.

AE2

AC3

Q5

Z1

Z2

experiment. If fat 3 – fat 4 had been, say, 17, we would have decla this difference significant and next tested fat 3 – fat 1 and fat 1 – fa against the D value for $a = 2$.

Whenever the highest and lowest of a group of means are found significantly different in this method, we declare that none of the memb of this group is distinguishable. This rule avoids logical contradiction the conclusions. The method is called *sequential* because the testing f lows a prescribed order or sequence.

Since protection against false claims of significance is obtained decreasing the ability to detect real differences, a realistic choice amc these methods requires a judgment about the relative seriousness of t two kinds of mistake. Duncan (32) has examined the type of policy th emerges if the investigator assigns relative costs to (i) declaring a sign cant result when the true difference is zero, (ii) declaring non-significan when there is a true difference, (iii) declaring a significant result in t wrong direction. His policy is designed to minimize the average cost mistakes in such verdicts of significance or non-significance. These cos are not necessarily monetary but might be in terms of utility or equit His optimum policy resembles an *LSD* rule with two notable difference In its simplest form, which applies when the number of treatments exceed 15 and $d.f.$ in s exceed 30, a difference between two means is declare significant if it exceeds $s_{\bar{D}} t_\infty \sqrt{F/(F-1)}$. The quantity t_∞ (not Student's depends on the relative costs assigned to wrong verdicts of significance c non-significance. If F is large, indicating that there are substantial differ ences among the population means of the treatments, $\sqrt{F/(F-1)}$ i nearly 1. The rule then resembles a simple *LSD* rule, but with the siz of the *LSD* determined by the relative costs. As F approaches 1, sugges ing that differences among treatment means are in general small, th difference required for significance becomes steadily larger, leading t greater caution in declaring differences significant. The F-value given b the experiment enters into the rule because F provides information as t whether real differences among treatment means are likely to be large o small. In Duncan's method, the investigator may also build into the rul his *a priori* judgment on this point.

In a large sampling experiment with four treatments, Balaam (33 compared (i) the *LSD* method, (ii) the revised *LSD* method in which no significant differences are declared unless F is significant, (iii) the Newman Keuls method (as well as other methods). Various sets of values were assigned to the population means μ_i, including a set in which all μ_i were equal. For each pair of means, a test procedure received a score of +1 if it ranked them correctly, a score 0 if it declared a significant difference when $\mu_i = \mu_j$ or found no difference when $\mu_i \neq \mu_j$, and a score −1 if it ranked the means in the wrong order. These scores were added over the six pairs of means.

When all μ_i were equal, the average scores were: *LSD*, 5.76: Revised *LSD*, 5.91; *NK*, 5.94. With three means equal, so that three of the six differences between pairs were equal and three unequal, average scores

were: *LSD*, 3.80; Revised *LSD*, 3.57; *NK*, 3.51. With more than 1 inequalities between pairs, average scores were: *LSD*, 1.92; Rev *LSD*, 1.73; *NK*, 1.63. To sum up for this section, no method is uniformly best. In critical situations, try to judge the relative costs of the two kinds of mistakes and be guided by these costs. For routine purposes, thoughtful use of either the *LSD* or the Newman-Keuls method should be satisfactory. Remember also Scheffé's test (p. 271) for a comparison that is picked out just because it looks large.

10.9—Shortcut computation using ranges. An easy method of testing all comparisons among means is based on the ranges of the samples (13). In the doughnut experiment, table 10.2.1, the four ranges are 39, 20, 30, 21; the sum is 110. This sum of ranges is multiplied by a factor taken from table 10.9.1. In the column for $a = 4$ and the row for $n = 6$, take the factor 0.95. Then

$$D' = \frac{(\text{Factor})(\text{Sum of Ranges})}{n} = \frac{(0.95)(110)}{6} = 17.4$$

D' is used like the D in the Q-test of the foregoing section. Comparing it with the six differences among treatments, we conclude, as before, that only the largest difference, 23, is significant.

TABLE 10.9.1
CRITICAL FACTORS FOR ALLOWANCES, 5% RISK*

Sample Size, n	Number of Samples, a								
	2	3	4	5	6	7	8	9	10
2	3.43	2.35	1.74	1.39	1.15	0.99	0.87	0.77	0.70
3	1.90	1.44	1.14	.94	.80	.70	.62	.56	.51
4	1.62	1.25	1.01	.84	.72	.63	.57	.51	.47
5	1.53	1.19	.96	.81	.70	.61	.55	.50	.45
6	1.50	1.17	.95	.80	.69	.61	.55	.49	.45
7	1.49	1.17	.95	.80	.69	.61	.55	.50	.45
8	1.49	1.18	.96	.81	.70	.62	.55	.50	.46
9	1.50	1.19	.97	.82	.71	.62	.56	.51	.47
10	1.52	1.20	.98	.83	.72	.63	.57	.52	.47

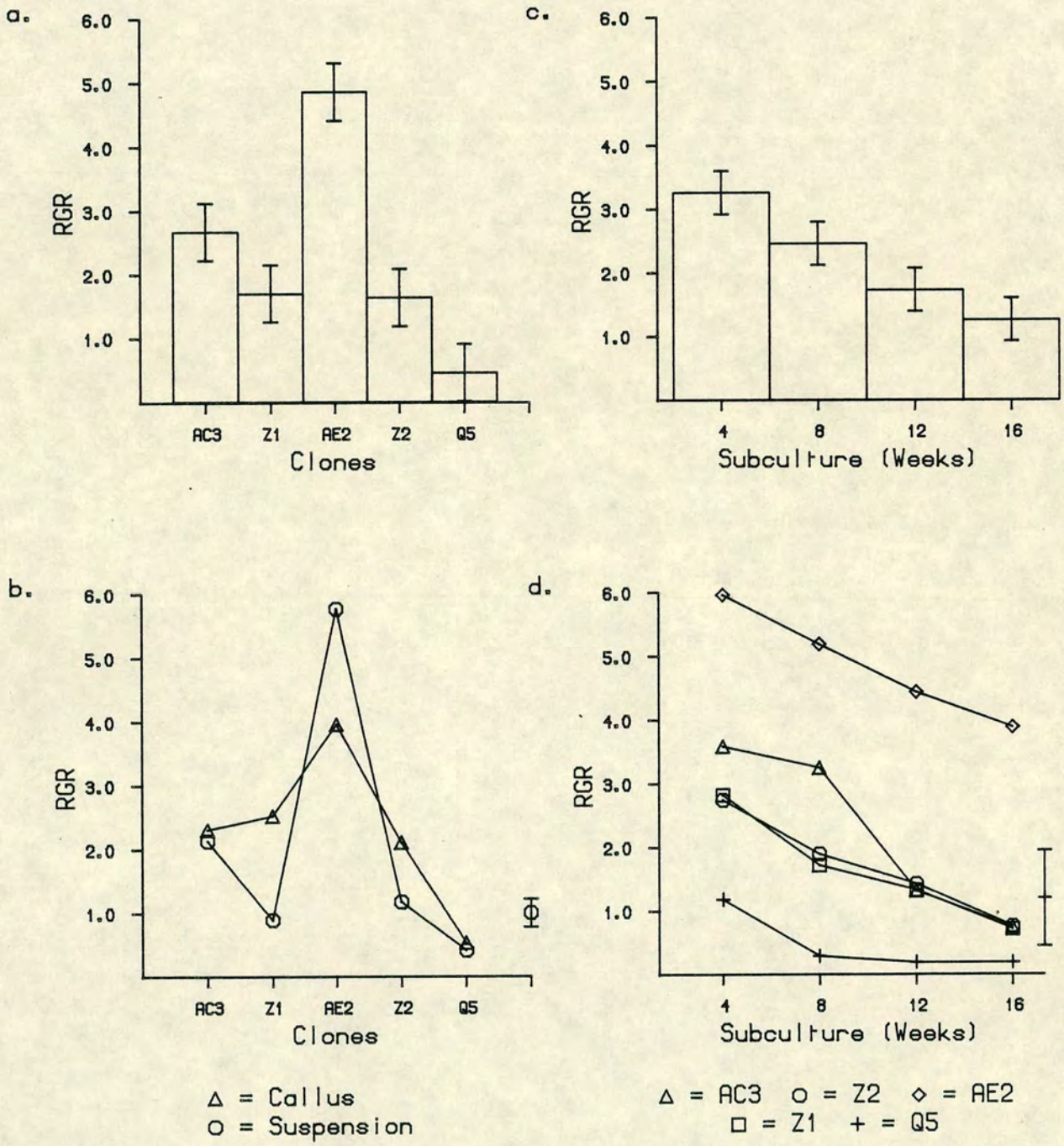
* Extracted from a more extensive table by Kurtz, Link, Tukey, and Wallace (13).

EXAMPLE 10.9.1—Using the shortcut method, examine all differences in the chick experiment of example 10.5.1 (p. 267). Ans. $D' = 49$. Same conclusions as for the Q method in example 10.8.2.

10.10—Model I. Fixed treatment effects. It is time to make a more formal statement about the assumptions underlying the analysis of variance for single classifications. A notation common in statistical papers is to use the subscript i to denote the class, where i takes on the values $1, 2, \ldots a$. The subscript j designates the members of a class, j going from 1 to n.

Figure 3.1.4

Comparison of relative growth rate (RGR) ($day^{-1}x10^{-2}$) of five clones maintained as callus or in suspension over sixteen weeks with a subculture and measurement every four weeks. The error bars represent the LSD values which were determined from the results of the analysis of variance shown in Table 1 of the Appendix.



c. Soluble phenolic compounds (SPCs) in the medium of the clones

c. i Among callus clones

Chromatograms showing the pattern of SPCs in the medium of a replicate culture from each of the five callus clones at the first and last subculture are shown in Fig. 3.1.5.

The patterns at the first subculture show that clone AE2 produced the greatest number of SPCs, of which P10, P11 and P12 were most prominent, although by the last subculture there was a drop in the number and type of SPCs produced by this clone (Fig. 3.1.5). Although present in lower amounts the pattern of SPCs produced by clone AC3 at the first subculture was similar to clone AE2, although P10 was not found, while similarly there was a drop in the amount and type of these SPCs by the last subculture (Fig. 3.1.5). Very few SPCs were produced by clones Z1, Z2 and Q5 at the first subculture, and by the last subculture none were detected in the medium of clones Z1 and Q5, and very few in Z2 (Fig. 3.1.5).

c ii. Among suspended clones

Chromatograms showing the pattern of SPCs in the medium of a replicate culture from each of the five clones in suspension at the first and last subculture are shown in Fig. 3.1.6.

As in the callus results it can be seen from the pattern of SPCs at the first subculture that the suspension cultures of clone AE2 produced the greatest number of SPCs, of which P7, P11 and P12 were most prominent, although by the last subculture the number and type of these SPCs dropped (Fig. 3.1.6). Although present in lower amounts the pattern of SPCs produced by clone AC3 at the first subculture was similar to clone AE2 (P7, P11 and P12 were again the most prominent) while there was a drop in the amount and type of SPCs produced by the last subculture (Fig. 3.1.6). Again like the callus clones there were very few SPCs found in the suspension cultures of clones Z1, Z2 and Q5 at either the first or last subculture, although as the patterns show there were greater amounts produced at the first (Fig. 3.1.6).

Figure 3.1.5

HPLC chromatograms of unknown $CHCL_3$ soluble phenolic compounds, as shown by number, in the medium of a replicate culture from each of five callus clones at the (a). first and (b). last subcultures of a sixteen week period. The spectra of these unknowns are shown in Fig 2.3.4.

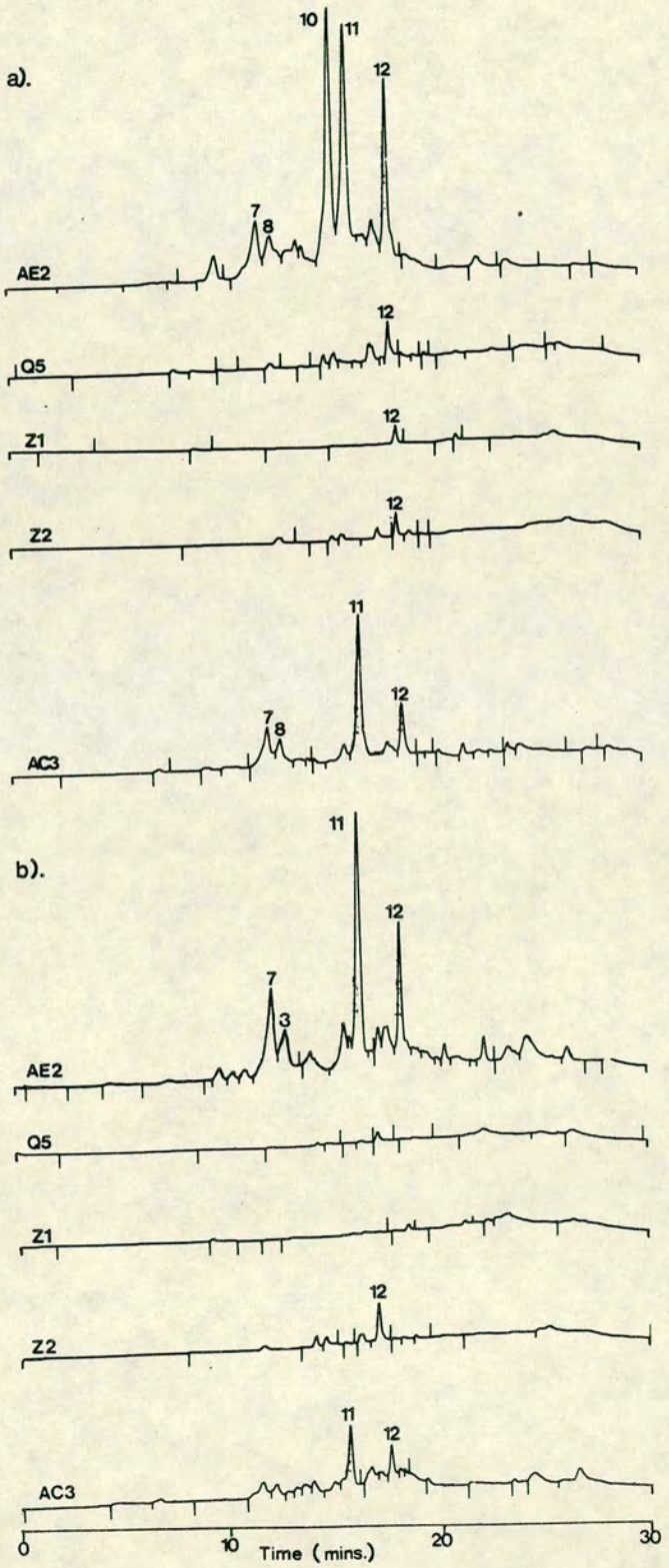
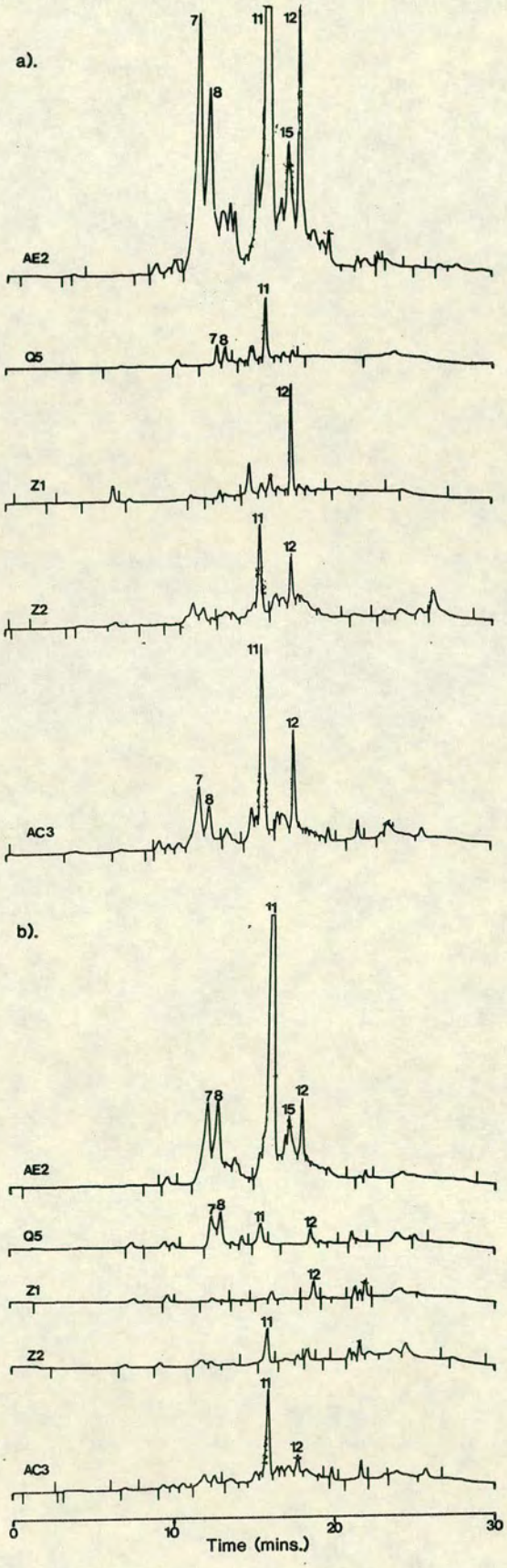


Figure 3.1.6

HPLC chromatograms of unknown $CHCL_3$ soluble phenolic compounds, as shown by number, in the medium of a replicate culture from each of five suspended clones at the (a). first and (b). last subcultures of a sixteen week period. The spectra of these unknowns are shown in Fig 2.3.4.



3.1.1.4 Conclusions on the comparisons of morphological and metabolic characteristics among clones isolated from a single suspension culture

Differences in the appearance, RGR and TCC were detected among the twenty callus clones at the four week analysis period. In a more detailed comparison, over the next four subcultures, clone AE2 remained yellow and friable with the highest RGR both in callus and, especially, suspension cultures and the greatest number of SPCs in both regimes. Clone AC3 became brown and aggregated which was accompanied by a significant drop in the RGR and a noticeable drop in the number of SPCs in both regimes, while the remaining three clones showed noticeably more unstable morphological and metabolic characteristics. Clones Z1 and Z2 were similar in that they both showed a rapid deterioration in appearance from yellow and friable to brown and aggregated growth with increasing subculture. This was accompanied by a low and falling RGR and low amounts of SPCs especially in suspension cultures. The brown and aggregated appearance of clone Q5 was detectable at the second subculture in both callus and suspension regimes, a result which was accompanied by the lowest mean RGR and a greatly reduced level of SPCs in both regimes over the sixteen week period.

To summarise, large differences in morphology and metabolism were detected among clones in both callus and suspension culture regimes which became more apparent with increasing subculture. Variation occurred with subculture as each one showed different rates of change with respect to their morphology and metabolism. It was decided to select clone AE2 for further study because of its high RGR, friability and pattern of phenolic accumulation.

3.1.2 Comparisons of morphological and metabolic characteristics within a clone

This second series of experiments comprises a study of the differences that exist within a clone at any one time and also among and within cultures derived from a clone over a period of time. It was conducted in two stages the first of which was a comparison of TCC and TPC at one time between replicate samples derived from each of three individual cultures. The second stage involved examining morphological and metabolic characteristics among and within daughter cultures derived from a single callus culture and maintained in different culture regimes.

3.1.2.1 Experimental procedure and design of experiments

a. Procedure for stage one (see Fig. 3.1.7)

The three callus cultures of the clone AE2, were carried over from the previous series of experiments. Each culture was subcultured after four weeks and the appearance, RGR, TPC and TCC were determined using the remaining cells. Three measurements of TCC and TPC were made from each of three separate samples from each of three cultures to test both the repeatability of these measurements and the differences within a culture at any one time.

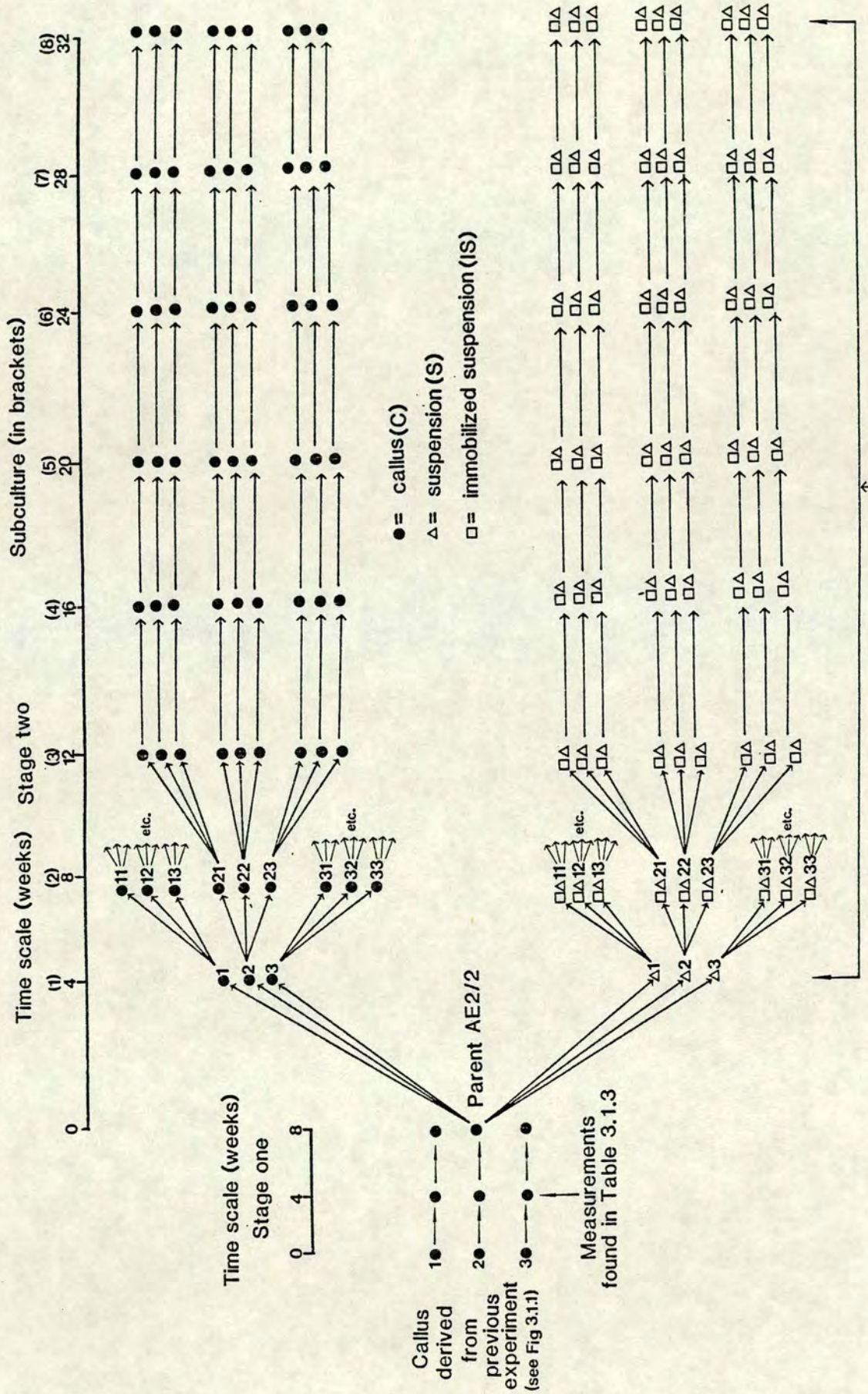
b. Procedure for stage two (see Fig. 3.1.7)

The callus culture which displayed the least variation in the sub-sample analysis was used for further study. This was chosen so that any differences in morphology and metabolism which developed could be attributed to events that occurred in this next experiment.

The culture was randomised and six cultures were obtained by subculture, three of the these cultures were maintained as callus (C) on agar and three as suspension (S) in liquid medium. All cultures were contained in 250 ml Erlenmeyer conical flasks for four weeks when three new callus cultures were established from each of the three parent cultures (C1,C2,C3), giving a total of nine individuals. At the same time six suspension cultures were established from each of the three parent cultures (S1,S2,S3).

Figure 3.1.7

Diagram showing the origins and relationships of experimental material, method of culture and times which data were collected.



From these six, three were maintained in suspension and three were immobilized, giving a total of nine suspension and nine immobilizing cultures. Using the remaining cell material, determinations of appearance, RGR, TCC and TPC were made, while an analysis of the SPCs in the medium was also carried out.

These nine callus, suspension or immobilizing cultures were maintained for four weeks when three new callus cultures were established from each of the nine parent cultures (C11-C33), giving a total of twenty-seven callus individuals. At the same time six suspension cultures were established from each of the nine parent suspension cultures (S11-S33). From these six, three were maintained in suspension and three were immobilized, giving a total of twenty-seven suspension and twenty-seven immobilizing cultures. Using the remaining cell material, the immobilized blocks and the residual media a determination of the morphological and metabolic characteristics was made.

One subculture of each callus culture, and two subcultures of each suspension culture, one of which was immobilized, were made every four weeks over the next twenty week period and after each subculture a determination of morphology and metabolism was carried out on the material that remained.

c. Analysis of results derived from stage two, (see Fig. 3.1.7)

The results of appearance, RGR, TCC, TPC and SPCs were used to determine differences among and within cultures derived from a single culture of clone AE2 within the callus, suspension and immobilized regimes. This involved taking every group of three daughter cultures and treating them as replicates from which a culture average (CA) score, for appearance and SPCs, or a culture mean (CM) value, for the numerical data, was found. Therefore, for example, there would be nine CA scores or CM values for each characteristic from each of the twenty-seven callus, suspension and immobilized cultures after the third subculture at twelve weeks. These cultures at twelve weeks were, therefore, replicates of the nine eight week old parent cultures, while cultures at eight weeks were replicates of the three four week old parent cultures. These could not be treated as true replicates as there was a four week period between their isolation and measurement, however this period was necessary for characteristics such as RGR to be determined.

Using the CA scores comparisons could be made, while the CM values provided the data for statistical analysis. In this respect, analysis of variance tests were used to test the significance of the variance ratio (VR) and hence that of the differences. The least significant difference (LSD) was then calculated to determine which CM values were significantly different from each other. Three analysis of variance tests were carried out;

1. The analysis of variance of the data obtained from residual material after the first set of subcultures at four weeks, showed the significance of the differences in CM values between the callus and suspension cultures. The three cultures in each regime were used as replicates.

2. The analysis of variance of the data obtained after the second set of subcultures at eight weeks, was used to determine (i). the significance of differences in CM values among cultures within callus, suspension and immobilized regimes, using the three groups of three replicate cultures in each regime, and (ii). the significance of differences in CM values among the three regimes using the nine replicate cultures in each regime.

3. The analysis of variance of the data obtained after the third and subsequent five subcultures at twelve weeks and onwards, was used to determine (i). the significance of differences in CM values among and within cultures in the three regimes with increasing subculture, using the nine groups of three replicate cultures from each subculture in each regime, and (ii). the significance of differences of CM values among the three regimes using the twenty-seven replicate cultures in each regime.

A comparative t-test was also employed to determine the significance of differences within cultures between the parent and daughter CM values at four, eight and twelve weeks. These statistical tests enable the following questions to be answered;

- Are there differences after subculture between parent and daughter cultures?

- Are there differences after subculture among daughter cultures derived from the original, four week and eight week parent cultures?

- Do differences increase or decrease with successive subculture?

3.1.2.2 Comparisons within three callus cultures of a clone at one time, results from stage one

It can be seen from the results presented in Table 3.1.3 that all three callus cultures of clone AE2 had high RGR values although they differed in appearance to some extent. The comparison among random samples taken from each culture revealed that although there were no obvious differences in TPC there were considerable differences among samples with respect to TCC (Table 3.1.3). However, although noticeable in AE2/1 and AE2/3 the differences in chlorophyll content were smaller in line AE2/2 and therefore, in view of this uniformity, this line was used as the source material for the next series of experiments involving subculture (Table 3.1.3, Fig 3.1.7).

3.1.2.3 Comparisons among and within cultures derived by subculture from a clone over a thirty-two week period, results from stage two

a. Appearance of the cultures

a. i. Among and within callus cultures

The CA scores presented in Table 3.1.4 show that the callus cultures turned mainly green and friable with some yellow cells at the first subculture after four weeks. This change was almost reversed at the next subculture as all three cultures, apart from C2 which remained mostly green, returned to being yellow and friable with some green cells (Table 3.1.4). At the next subculture, after twelve weeks, the nine cultures were predominantly yellow and friable, with some having more green or white cells than others, giving some a variegated appearance (Table 3.1.4, Fig 3.1.8a.). This variegation became more noticeable over the remaining subcultures, so by the end of the experiment although all cultures were mainly yellow, there were at least two other types of coloured cells present (Table 3.1.4, Fig 3.1.8b.).

a. ii. Among and within suspension cultures

The CA scores presented in Table 3.1.5 show that the suspension cultures turned mainly green and friable with some yellow cells at the first subculture after four weeks. However, this change was almost completely reversed at the next subculture when all three cultures returned to being mainly yellow and friable with green cells, although there was some aggregation in S1 (Table 3.1.5).

Table 3.1.3

Appearance, relative growth rate (RGR)($day^{-1}x10^{-2}$) and replicate measurements of total chlorophyll content (TCC) (µg g DW^{-1}) and total protein content (TPC) (mg g DW^{-1}), including mean ($\bar{x}$) and standard deviation (sd.), of three callus cultures of clone AE2.

Clone	Appearance	RGR	TCC	$\bar{x}$	sd.	TPC	$\bar{x}$	sd.
AE2/1	Y,F	5.68	12.8 0.00 6.90	6.57	(6.40)	15.8 14.3 13.6	14.5	(1.17)
AE2/2	Y(G),F	6.72	128.1 134.2 119.7	127.2	(7.3)	14.4 15.3 13.8	14.5	(0.75)
AE2/3	Y(G),F	5.93	142.7 96.1 101.1	113.1	(25.6)	12.8 15.3 14.6	14.1	(1.25)

Y=Yellow

G=Green

B=Brown

W=White

F=Friable

A=Aggregated

V=Variegated

Y(G) means culture mainly yellow with the presence of a small amount of cells of one other colour, in this case green.

Y(V) means culture mainly yellow with the presence of small amounts of other coloured cells (in this case white, green or brown) giving the culture a variegated appearance.

F(A) means culture mostly friable with small amounts of aggregated cells.

Table 3.1.4

CA score for appearance of parent and derived callus cultures (C) at each subculture during a thirty-two week period. Each score is an average of three sets of observations. Numbers represent the culture numbers.

Parent Y(G),Fr			**Subculture (Weeks)**					
			Wk12	**Wk16**	**Wk20**	**Wk24**	**Wk28**	**Wk32**
	Wk8	**11**	Y(G),F	Y(G),F	Y(V),F	Y(V),F	Y(V),F	Y(V),F
	1 Y(G),F	**12**	Y(G),F	Y(G),F	Y(V),F	Y(V),F	Y(V),F	Y(V),F
		13	Y(V),F	Y(V),F	Y(V),F	Y(V),F	Y(V),F	Y(V),F
Wk4		**21**	Y(V),F	Y(G),F	Y(V),F	Y(V),F	Y(V),F	Y(V),F
G(Y),F	**2** G(Y),F	**22**	Y(G),F	Y(G),F	Y(G),F	Y(G),F	Y(G),F	Y(V),F
		23	Y(V),F	Y(V),F	Y(G),F	Y(G),F	Y(G),F	Y(V),F
		31	Y(V),F	Y(V),F	Y(V),F	Y(V),F	Y(V),F	Y(V),F
	3 Y(G),F	**32**	Y(G),F	Y(G),F	Y(G),F	Y(G),F	Y(V),F	Y(V),F
		33	Y(V),F	Y(V),F	Y(V),F	Y(V),F	Y(B),F	Y(B),F

Y=Yellow

G=Green

B=Brown

W=White

F=Friable

A=Aggregated

V=Variegated

Y(G) means culture mainly yellow with the presence of a small amount of cells of one other colour, in this case green.

Y(V) means culture mainly yellow with the presence of small amounts of other coloured cells (in this case white, green or brown) giving the culture a variegated appearance.

F(A) means culture mostly friable with small amounts of aggregated cells.

Figure 3.1.8

1. The appearance of a replicate callus culture from each of three parent cultures (C11,C13,C22) at the third subculture after twelve weeks.

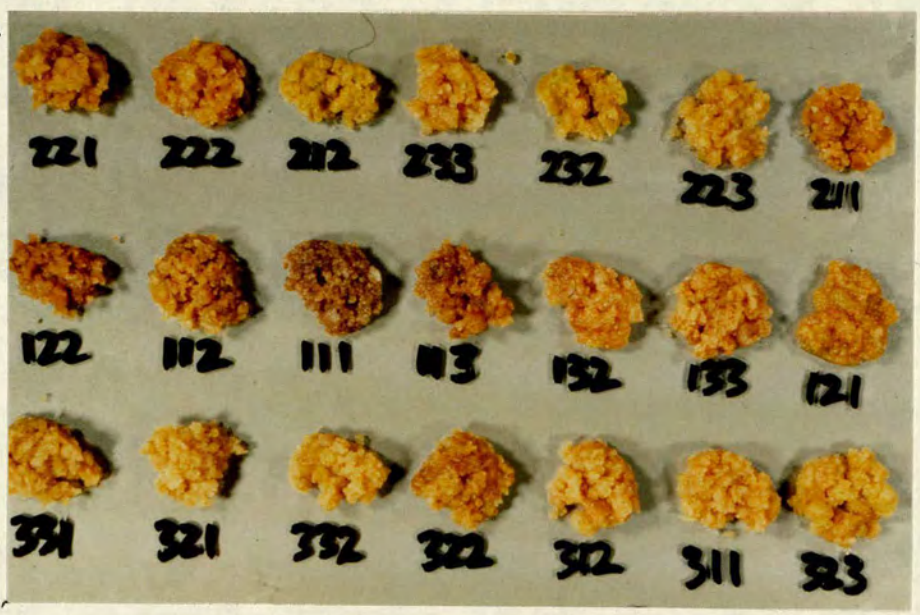
2. The appearance of a replicate callus culture from each of three parent cultures (C23,C31,C33) at the third subculture after twelve weeks.

3. The appearance of cell material from twenty-seven replicate callus cultures (ie; C221 was derived from the C22 parent) at the sixth subculture after twenty-four weeks.

1.a.

2.a.

3.b.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At the next subculture, after twelve weeks, all the nine cultures apart from S32, remained predominantly yellow and friable with green cells in those from the eight week parent culture S1, green and white cells from S2 and brown cells in those from S3 (Table 3.1.5, Fig 3.1.9a.). The cultures derived from the four week parent culture S1 were more aggregated and were notable for the stability of their appearance (Table 3.1.5). These differences among the cultures which had appeared by twelve weeks persisted throughout most of the later subcultures (Table 3.1.5, Fig 3.1.9b.). Brown cells appeared at different times, although they were very noticeable in S32 and S33, and once present the brown colour was never lost (Table 3.1.5).

a. iii. Among and within immobilized suspension cultures

The CA scores presented in Table 3.1.6 show that the immobilized suspension cultures were mainly yellow, with some green cells present in IS1 after the subculture at eight weeks (Table 3.1.6). At twelve weeks, the nine cultures were all yellow, apart from IS32 and IS33 which were brown, with, in the case of cultures derived from the eight week parent cultures S1 and S2, small areas of green or white cells or both (Table 3.1.6, Fig 3.1.9c.). From twelve weeks onwards the cultures IS32 and IS33 continued to be brown and aggregated, whereas the others continued to be yellow and variegated. Those cultures derived from S2 were notable for having a greater number of differently coloured cells in their cultures (Table 3.1.6).

Table 3.1.5

CA score for appearance of parent and derived suspension cultures (S) at each subculture during a thirty-two week period. Each score is an average of three sets of observations. Numbers represent the culture numbers.

Parent Y(G),Fr		**Subculture (Weeks)**					
		Wk12	**Wk16**	**Wk20**	**Wk24**	**Wk28**	**Wk32**
	Wk8	**11** Y(G),F(A)	Y(G),F(A)	Y(G),F(A)	Y(G),F(A)	Y(G),F(A)	Y(B),F(A)
	1 Y(G),F(A)	**12** Y(G),F(A)	Y(G),F(A)	Y(G),F(A)	Y(G),F(A)	Y(B),F(A)	Y(M),F
		13 Y(G),F(A)	Y(G),F	Y(G),F(A)	Y(G),F(A)	Y(G),F(A)	Y(B),F(A)
Wk4		**21** Y(V),F(A)	Y(V),F(A)	Y(W),F(A)	Y(V),F(A)	Y(B),F(A)	B(Y),F(A)
G(Y),F	**2** Y(G),F	**22** Y(V),F	Y(V),F	Y(V),F(A)	Y(V),F(A)	Y(V),F(A)	Y(V),Fr
		23 Y(V),F(A)	Y(V),F(A)	Y(V),F(A)	Y(B),F(A)	Y(B),F(A)	Y(B),F(A)
		31 Y(G),F	Y(G),F	Y(V),F	Y(V),F	Y(B),F	Y(B),F
	3 Y(G),F	**32** B(Y),F	B(Y),F(A)	B,F(A)	B,F(A)	B,F(A)	B,F(A)
		33 Y(V),F	B,F(A)	B,F(A)	B,F(A)	B,F(A)	B,F(A)

Y=Yellow

G=Green

B=Brown

W=White

F=Friable

A=Aggregated

V=Variegated

Y(G) means culture mainly yellow with the presence of a small amount of cells of one other colour, in this case green.

Y(V) means culture mainly yellow with the presence of small amounts of other coloured cells (in this case white, green or brown) giving the culture a variegated appearance.

F(A) means culture mostly friable with small amounts of aggregated cells.

Table 3.1.6

CA score for appearance of parent and derived immobilized suspension cultures (IS) at each subculture during a thirty-two week period. Each score is an average of three sets of observations. Numbers represent the culture numbers.

Parent Y(G),Fr		**Subculture (Weeks)**				
	Wk12	**Wk16**	**Wk20**	**Wk24**	**Wk28**	**Wk32**
Wk8	**11** Y(V),I	Y(V),I	Y(V),I	Y(V),I	Y(V),I	Y(V),A
1 Y(G),I	**12** Y(V),I	Y(V),I	Y(G),I	Y(G),I	Y(V),I	Y(V),I
	13 Y(G),I	Y(V),I	Y(G),I	Y(G),I	Y(V),I	Y(V),I
	21 Y(G),I	Y(G),I	Y,I	Y(V),I	Y(B),I	B(Y),A
2 Y,I	**22** Y(W),I	Y(W),I	Y,I	Y,I	Y(B),I	B(Y),A
	23 Y(V),I	Y(V),I	Y,I	Y(B),I	Y(B),I	B(Y),A
	31 Y(G),I	Y(G),I	Y(G),I	Y(G),I	Y,I	Y,A
3 Y,I	**32** B(G),I	B,I	B,A	B,A	B,A	B,A
	33 B(Y),I	B,I	B,I	B,A	B,A	B,A

Y=Yellow

G=Green

B=Brown

W=White

F=Friable

A=Aggregated

V=Variegated

I=Immobilized

Y(G) means culture mainly yellow with the presence of a small amount of cells of one other colour, in this case green.

Y(V) means culture mainly yellow with the presence of small amounts of other coloured cells (in this case white, green or brown) giving the culture a variegated appearance.

F(A) means culture mostly friable with small amounts of aggregated cells.

a. iv. Comparison of appearance among regimes and summary of results

At the first subculture there was a noticeable drop in the number of green cells in the callus and suspension cultures and by twelve weeks the cultures within all three regimes were mainly yellow in colour with one or two additional colours the amounts of which varied considerably among and within the regimes. Callus cultures were more stable in appearance but both the suspension and immobilized cultures showed some early and apparently irreversible differentiation from yellow to brown, particularly in S and IS32 and 33. Callus and suspension regimes were similar in having predominantly friable cultures. However, aggregated cells were more common in the suspension cultures particularly with increasing subculture, although it did not appear to restrict immobilization in subsequent cultures.

To summarise, differences in appearance exist among cultures derived from the one callus culture although they are restricted only to the secondary colour or texture. More noticeable were the differences within the cultures after twelve weeks as shown by the increasing variegation in all regimes. Nevertheless, these results do show that subculture can result in the isolation of morphologically different cultures in, for example, the aggregation that arose in suspension cultures derived from parent culture S1 or the browning in suspension cultures of S3.

Figure 3.1.9

1. The appearance of a replicate suspension culture from each of three parent cultures (S11,S22,S32) at the third subculture after twelve weeks.

2. The appearance of cell material from eighteen replicate suspension cultures (ie; S233 was derived from the S23 parent) at the sixth subculture after twenty-four weeks.

3. The appearance of cell material from nine replicate immobilized suspension cultures (ie; I122 was derived from the S12 parent) at the third subculture after twelve weeks.

1.a.


250
200
150
100

2.b.


233
312
212
222
113
313
311
132
133
223
122
131
221
131
132
112
213
111

3.c.


122
133
132
233
231
221
312
313
322

b. Relative growth rate (RGR) of the cultures

b. i. Among and within callus cultures

It can be seen from the CM values in Table 3.1.7 that there were no significant differences in RGR among the three callus cultures at eight weeks or among the nine from twelve and thirty-two weeks. Furthermore, there were no significant differences in RGR within the cultures from four to twelve weeks although significant differences were found within the cultures from twelve to thirty-two weeks (Table 3.1.7). Over this latter period there was a significant drop in the RGR at sixteen and thirty-two weeks; however, this fall at the first of these two subculture times was due mainly to the considerable drop in the RGR of culture C33 (Table 3.1.7).

b. ii. Among and within suspension cultures

The CM values in Table 3.1.8 show that although there were no significant differences in RGR among the three suspension cultures at eight weeks, there were significant differences among the nine from twelve to thirty-two weeks. Furthermore, there were significant differences in the RGR within cultures from twelve and thirty-two weeks although not over the initial four to twelve weeks (Table 3.1.8).

The reason for the differences among the nine cultures was due to the significantly lower RGR of cultures S32 and S33 from the subculture at sixteen weeks onwards (Table 3.1.8). There was also a significant drop in the RGR within cultures at the twenty-eight and thirty-two week subculture times, however inspection of the CM values within the S32 and S33 cultures show that this fall occurred much earlier in the experiment (Table 3.1.8).

b. iii. Among and within immobilized suspension cultures

It can be seen in Table 3.1.9 that although there were no significant differences in RGR among the three immobilized suspension cultures at eight weeks, there were significant differences among the nine from twelve to thirty-two weeks. Furthermore, there were no significant differences in RGR within the cultures from four to twelve weeks, although significant differences were found within cultures from twelve to thirty-two weeks (Table 3.1.9).

Table 3.1.7

CM values for relative growth rate (RGR) ($day^{-1}x10^{-2}$) of parent and derived callus cultures (C) at each subculture during a thirty-two week period. Final column presents the overall means for the nine cultures and the final row presents the overall means at the six subculture times. Each result is the mean of three separate measurements. Numbers in brackets represent the culture numbers.

Parent 6.72		**Subculture (Weeks)**						
		Wk12	**16**	**20**	**24**	**28**	**32**	**Overall Means**
	Wk8	**(11)** 8.89	6.31	6.17	5.69	3.81	2.26	5.52
	(1) 7.54	**(12)** 7.16	6.78	5.28	7.36	6.27	4.04	6.15
		(13) 8.06	6.55	6.50	7.10	6.46	4.15	6.47
Wk4		**(21)** 8.36	6.71	6.76	7.65	7.34	3.71	6.75
7.01	**(2)** 8.55	**(22)** 8.47	7.16	7.53	7.63	7.02	4.09	6.98
		(23) 8.53	6.65	6.51	7.49	7.71	5.70	7.10
		(31) 8.63	6.02	6.58	7.30	6.29	1.78	6.10
	(3) 9.49	**(32)** 7.55	6.12	6.78	7.67	7.29	4.33	6.62
	LSD 2.47	**(33)** 7.22	3.96	6.95	7.55	6.99	3.88	6.09
	Overall means	8.09	6.25	6.56	7.27	6.57	3.77	
				LSD 1.21				

LSD 1.66

The appropriate LSD values were used to compare differences among the three eight week culture values, and among the nine culture and six subculture values from twelve to thirty-two weeks. These were obtained from an analysis of variance the results of which are presented in Table 2 of the Appendix. The results of the comparative t-test between values from four to twelve weeks are shown in Table 4 of the Appendix.

Table 3.1.8

CM values for relative growth rate (RGR) ($day^{-1}x10^{-2}$) of parent and derived suspension cultures (S) at each subculture during a thirty-two week period. Final column presents the overall means for the nine cultures and the final row presents the overall means at the six subculture times. Each result is the mean of three separate measurements. Numbers in brackets represent the culture numbers.

Parent 6.72			**Subculture (Weeks)**					
		Wk12	**16**	**20**	**24**	**28**	**32**	**Overall Means**
	Wk8	**(11)** 7.69	7.47	6.99	5.95	4.18	0.00	5.38
	(1) 7.41	**(12)** 8.46	6.29	6.92	5.83	3.72	2.55	5.63
		(13) 8.34	6.54	6.75	6.17	3.86	2.99	5.77
Wk4		**(21)** 6.65	7.47	4.43	5.51	1.29	1.41	4.45
7.57	**(2)** 7.41	**(22)** 8.16	6.86	5.95	6.54	3.26	2.52	5.55
		(23) 8.24	6.97	5.96	6.14	1.58	1.35	5.02
		(31) 7.90	6.55	4.29	6.35	3.49	1.03	4.94
	(3) 7.90	**(32)** 6.35	2.29	1.27	2.08	1.15	0.00	1.98
	LSD 2.47	**(33)** 9.08	2.29	1.11	1.77	1.15	0.00	2.38
	Overall means	7.87	5.85	4.88	5.15	2.63	1.32	
				LSD 1.22				

LSD 1.66

The appropriate LSD values were used to compare the differences among the three eight week culture values, and among the nine culture and six subculture values from twelve to thirty-two weeks. These were obtained from an analysis of variance the results of which are presented in Table 2 of the Appendix. The results of the comparative t-test between values from four to twelve weeks are shown in Table 4 of the Appendix.

The differences among the nine cultures were due to the high RGR values of cultures derived from the eight week old parent culture S1 (IS11, IS12, IS13), and the significantly lower RGR of cultures derived from S3 (mainly IS32, IS33) (Table 3.1.9). There was also a drop in the RGR within cultures at twenty-four weeks and the remaining subculture times, however this fall within the IS32 and IS33 cultures occurred much earlier in the experiment (Table 3.1.9).

b. iv. Comparison of RGR among regimes and summary of results

The CM values for regimes presented in Table 3.1.10 show no significant differences in RGR among regimes at the subcultures after four and eight weeks. However, from twelve to thirty-two weeks, and especially over the latter half of this period, the callus cultures had a consistently greater RGR than either suspension or immobilized suspension cultures which was shown by the significantly higher overall CM value for this period (Table 3.1.10). The immobilized cultures had the lowest CM value for RGR overall and at every subculture in the experiment.

To summarise, differences in RGR were not found among or within cultures derived from a single culture and maintained in different regimes, until after the third subculture. In this respect, the cultures maintained in the suspension or immobilized suspension regimes showed a decline in RGR with increasing subculture whereas the RGR of the callus cultures was high and stable until the very last subculture. One noticeable feature of this experiment was the rapid decline of RGR in two of the nine eight week derived suspension cultures which caused significant differences to arise among cultures overall and at individual subculture times. This could have been a result of differences which were present in the original parent culture but were not revealed until after a later subculture.

Table 3.1.9

CM values for relative growth rate (RGR) ($day^{-1}x10^{-2}$) of parent and derived immobilized suspension cultures (IS) at each subculture during a thirty-two week period. Final column presents the overall means for the nine cultures and the final row presents the overall means at the six subculture times. Each result is the mean of three separate measurements. Numbers in brackets represent the culture numbers.

Parent 6.72		**Subculture (Weeks)**						
	Wk12	**16**	**20**	**24**	**28**	**32**	**Overall Means**	
Wk8	**(11)** 5.40	4.50	5.47	1.78	2.44	0.00	3.26	
(1) 5.87	**(12)** 5.11	5.47	6.24	2.84	1.53	2.66	4.02	
	(13) 4.18	4.31	3.95	2.29	2.36	1.65	3.12	
	(21) 3.67	3.04	4.99	1.24	1.91	0.00	2.47	
(2) 5.01	**(22)** 5.79	1.30	3.83	2.79	1.42	0.00	2.52	**LSD 1.39**
	(23) 4.57	2.83	2.51	1.70	0.83	0.00	2.07	
	(31) 5.22	0.00	4.16	1.81	1.70	0.00	2.15	
(3) 5.43	**(32)** 4.11	2.74	0.00	0.00	0.00	0.00	1.14	
LSD 2.47	**(33)** 3.99	2.67	1.85	0.00	0.00	0.00	1.42	
Overall means	3.55	3.01	3.67	1.60	1.35	0.48		
			LSD 1.03					

The appropriate LSD values were used to compare the differences among the three eight week culture values, and among the nine culture and six subculture values from twelve to thirty-two weeks. These were obtained from an analysis of variance the results of which are presented in Table 2 of the Appendix. The results of the comparative t-test between values from eight to twelve weeks are shown in Table 4 of the Appendix.

Table 3.1.10

Comparison of the mean relative growth rate (RGR) ($day^{-1}x10^{-2}$) of all callus, suspension and immobilized suspension cultures at each subculture during a thirty-two week period. Final column presents the overall means for each regime from twelve to thirty-two weeks.

	Subculture (Weeks)								
Regime	**Wk4**	**8**	**12**	**16**	**20**	**24**	**28**	**32**	**Overall Means**
Callus	7.01	8.52	8.09	6.25	6.56	7.27	6.57	3.77	6.42
Suspension	7.57	7.57	7.87	5.85	4.59	5.15	2.63	1.32	4.56
Immobilized-Suspension		5.44	3.54	3.02	3.67	1.60	1.35	0.48	2.27
LSD values	**3.24**	**1.29**							**0.40**

The appropriate LSD values were obtained from three individual analysis of variance tests shown in Tables 2 and 3 of the Appendix and were used to compare the differences among the overall means at four, eight and from twelve to thirty-two weeks.

c. Total chlorophyll content (TCC) of the cultures

c. i. Among and within callus cultures

It can be seen from the CM values in Table 3.1.11 that there were no significant differences in TCC among the three callus cultures at eight weeks or among the nine from twelve to twenty-eight weeks. However, although there were no significant differences in TCC within cultures from eight to twenty-eight weeks, there was found to be a significant drop in the TCC in all three cultures at the eight week subculture (Table 3.1.11).

c. ii. Among and within suspension cultures

The CM values in Table 3.1.12 show that although there were no significant differences in TCC among the three suspension cultures at eight weeks there were significant differences among the nine cultures from twelve to twenty-eight weeks. Furthermore, significant differences were found in TCC within the cultures over the subculture period from four to eight, and from twelve to twenty-eight weeks (Table 3.1.12).

The differences among the nine cultures were due to the significantly lower TCC in three of the nine cultures over this period, two of these cultures (S32 and S33) were derived from S3 and the other (S21) was derived from S2 (Table 3.1.12). There was a significant drop in the TCC of all three cultures at the eight week subculture, following this the TCC fell with increasing subculture in all cultures from twelve weeks onwards, although only after sixteen weeks was the drop found to be significant (Table 3.1.12). This fall in TCC over the twelve to twenty-eight week period was more noticeable in the cultures derived from the eight week old parent culture S3, especially at twenty weeks (Table 3.1.12).

c. iii. Among and within immobilized suspension cultures

It can be seen from the CM values that although there were no significant differences in TCC among the three immobilized suspension cultures at eight weeks there were significant differences among the nine cultures from twelve to twenty-eight weeks (Table 3.1.13). Furthermore, significant differences were found in TCC within the cultures from twelve to twenty-eight weeks (Table 3.1.13).

Table 3.1.11

CM values for total chlorophyll content (TCC) (µg g DW^{-1}) of parent and derived callus cultures (C) at each subculture during a twenty-eight week period. Final column presents the overall means for the nine cultures and the final row presents the overall means at the five subculture times. Each result is the mean of three separate measurements. The numbers in brackets represent the culture numbers.

Parent 127.2		**Subculture (Weeks)**						
		Wk12	**16**	**20**	**24**	**28**	**Overall Means**	
	Wk8	**(11)** 24.7	14.9	26.6	6.60	5.40	15.6	
	(1) 101.0	**(12)** 33.0	21.3	38.0	11.6	25.7	26.0	
		(13) 47.7	22.2	29.3	7.80	52.1	31.8	
Wk4		**(21)** 31.5	20.2	48.7	14.5	60.0	35.0	**LSD**
259.0	**(2)** 108.0	**(22)** 15.6	20.6	48.6	34.7	24.3	28.8	**28.6**
		(23) 33.2	41.0	45.0	84.4	30.1	46.6	
		(31) 27.8	11.8	24.9	33.9	37.2	27.1	
	(3) 69.0	**(32)** 17.3	23.0	36.1	34.0	50.9	32.3	
	LSD 116.4	**(33)** 55.0	13.8	18.0	67.4	32.4	37.3	
	Overall means	32.0	21.0	35.1	43.9	35.3		
				LSD 19.7				

The appropriate LSD values were used to compare differences among the three eight week culture values, and among the nine culture and five subculture values from twelve to twenty-eight weeks. These were obtained from an analysis of variance the results of which are presented in Table 5 of the Appendix. The results of a comparative t-test between values from four to twelve weeks are shown in Table 7 of the Appendix.

Table 3.1.12

CM values for total chlorophyll content (TCC) (µg g DW^{-1}) of parent and derived suspension cultures (S) at each subculture during a twenty-eight week period. Final column presents the overall means for the nine cultures and the final row presents the overall means at the five subculture times. Each result is the mean of three separate measurements. The numbers in brackets represent the culture numbers.

Parent 127.2		**Subculture (Weeks)**						
		Wk12	**16**	**20**	**24**	**28**	**Overall Means**	
	Wk8	**(11)** 26.3	8.80	11.2	12.4	8.10	13.3	
	(1) 24.0	**(12)** 30.4	8.45	9.40	34.0	14.8	17.7	
		(13) 16.7	8.20	11.0	0.00	0.00	7.10	
Wk4		**(21)** 26.0	11.0	0.00	7.40	0.00	9.08	
298.0	**(2)** 77.0	**(22)** 23.1	11.6	7.40	0.00	0.00	8.40	**LSD 12.3**
		(23) 37.0	13.2	7.30	0.00	0.00	11.4	
		(31) 18.3	11.5	0.00	5.00	0.00	7.00	
	(3) 20.0	**(32)** 0.00	5.40	0.00	0.00	0.00	1.10	
	LSD 116.4	**(33)** 8.20	11.6	0.00	0.00	0.00	4.00	
	Overall means	20.0	11.0	5.10	6.50	2.50		
				LSD 8.49				

The appropriate LSD values were used to compare differences among the three eight week culture values and among the nine culture and five subculture values from twelve to twenty-eight weeks. These were obtained from an analysis of variance the results of which are presented in Table 5 of the Appendix. The results of a comparative t-test between values from four to twelve weeks are shown in Table 7 of the Appendix.

The significant differences among the nine cultures were due only to the difference between cultures IS22 and IS23, the former having a greatly reduced TCC (Table 3.1.13). There was a significant drop in the TCC of all nine cultures at sixteen weeks and in those derived from the eight week parent cultures S2 and S3 at twenty weeks (Table 3.1.13). The drop in TCC was so marked that chlorophyll could not be detected in any of the nine cultures by the last subculture (Table 3.1.13).

c. iv. Comparison of TCC among regimes and summary of results

The CM values for regimes presented in Table 3.1.14 show significant differences in TCC among regimes at four, eight and from twelve to twenty-eight weeks. At the subculture at four weeks a high TCC was found in both callus and suspension regimes, whereas cultures in the immobilized suspension regime accumulated significantly lower amounts of chlorophyll (Table 3.1.14). At eight weeks the TCC of all cultures dropped, however, by this stage only the callus cultures had a significantly greater TCC than the immobilized suspension cultures (Table 3.1.14). From twelve to twenty-eight weeks the callus cultures had a consistently greater TCC than either suspension or immobilized suspension cultures as shown by the significantly greater overall CM value for this period (Table 3.1.14). The immobilized suspension cultures had the lowest CM value for TCC overall and at every subculture in the experiment (Table 3.1.14).

To summarise, although there were no significant differences among cultures in any regime over the first twelve weeks there was a significant and consistent drop in TCC in the cultures of all regimes at eight weeks. The differences among the regimes over the twelve to twenty-eight week period was a result of a consistently high and stable TCC in callus cultures, and the drop in TCC in suspension and immobilized suspension cultures over this period. In this respect the cultures derived from the eight week old parent cultures S2 and S3 showed a rapid decline in chlorophyll accumulation from twenty weeks which caused significant differences both within and among cultures of the suspension and immobilized suspension regimes.

Table 3.1.13

CM values for total chlorophyll content (TCC) (µg g DW^{-1}) of parent and derived immobilized suspension cultures (IS) at each subculture during a twenty-eight week period. Final column presents the overall means for the nine cultures and the final row presents the overall means at the five subculture times. Each result is the mean of three separate measurements. The numbers in brackets represent the culture numbers.

Parent 127.2	**Subculture (Weeks)**						
	Wk12	**16**	**20**	**24**	**28**	**Overall Means**	
Wk8	**(11)** 6.70	5.30	6.20	6.10	0.00	4.80	
(1) 11.7	**(12)** 1.10	0.00	14.6	12.0	0.00	5.50	
	(13) 12.2	8.50	3.60	0.00	0.00	4.90	
	(21) 17.5	3.10	0.00	0.00	0.00	4.10	
(2) 6.14	**(22)** 2.80	2.30	0.00	0.00	0.00	1.10	**LSD 6.69**
	(23) 30.7	12.4	5.60	0.00	0.00	9.70	
	(31) 10.7	9.30	0.00	0.00	0.00	3.90	
(3) 5.20	**(32)** 8.70	4.90	0.00	0.00	0.00	2.70	
LSD 116.4	**(33)** 10.7	10.1	0.00	0.00	0.00	4.20	
Overall means	11.3	6.20	3.30	2.10	0.00		
			LSD 4.62				

The appropriate LSD values were used to compare differences among the three eight week culture values, and among the nine culture and five subculture values from twelve to twenty-eight weeks. These were obtained from an analysis of variance the results of which are presented in Table 5 of the Appendix. The results of the comparative t-test between values from eight to twelve weeks are shown in Table 7 of the Appendix.

Table 3.1.14

Comparison of the mean total chlorophyll content (TCC) (µg g DW^{-1}) of all callus, suspension and immobilized suspension cultures at each subculture during a twenty-eight week period. Final column presents the overall means from twelve to twenty-eight weeks.

	Subculture (Weeks)							
Regime	**Wk4**	**8**	**12**	**16**	**20**	**24**	**28**	**Overall Means**
Callus	259.0	92.1	31.7	20.9	35.1	43.9	35.3	33.4
Suspension	298.0	40.3	20.6	11.0	5.10	6.50	2.50	8.70
Immobilized-Suspension		7.60	11.2	6.20	3.30	2.10	0.00	4.56
LSD values	**68.7**	**57.2**						**4.62**

The three LSD values were obtained by three individual analysis of variance tests shown in Tables 5 and 6 of the Appendix and were used to compare the differences among the overall means at four, eight and between twelve and twenty-eight weeks.

d. Total protein content (TPC) of the cultures

d. i. Among and within callus cultures

The CM values presented in Table 3.1.15 show that there were no significant differences in the TPC among the three callus cultures at eight weeks or among the nine from twelve and thirty-two weeks. Furthermore there were no significant differences within cultures from four to twelve weeks or during the period from twelve to thirty-two weeks, although there was a noticeable drop in TPC at the last subculture in most of the nine cultures (Table 3.1.15).

d. ii. Among and within suspension cultures

It can be seen from the CM values in Table 3.1.16 that although there were no significant differences among the three suspension cultures at eight weeks there were differences among the nine from twelve to thirty-two weeks (Table 3.1.16). Furthermore, there were also significant differences found in the TPC within cultures from twelve to thirty-two weeks although not from four to twelve weeks (Table 3.1.16).

The differences among the nine cultures were accounted for by the significantly lower TPC in cultures S32 and S33 from the subculture at sixteen weeks onwards (Table 3.1.16). There was also a significant drop in the TPC within the nine cultures at sixteen and thirty-two weeks; however, this was more significant in the cultures S32 and S33 (Table 3.1.16).

d. iii. Among and within immobilized suspension cultures

It can be seen from the CM values in Table 3.1.17 that although there were no significant differences among the three immobilized suspension cultures at eight weeks there were differences among the nine from twelve and thirty-two weeks. Furthermore, significant differences were found within the cultures from twelve to thirty-two weeks although not over the initial four to twelve weeks (Tables 3.1.17).

Table 3.1.15

CM values for total protein content (TPC) (mg g DW^{-1}) of parent and derived callus cultures (C) at each subculture during a thirty-two week period. Final column presents the overall means for the nine cultures and the final row presents the overall means at the six subculture times. Each result is the mean of three separate measurements. The numbers in brackets represent the culture numbers.

Parent 15.3		**Subculture (Weeks)**						
		Wk12	**16**	**20**	**24**	**28**	**32**	**Overall Means**
	Wk8	**(11)** 15.8	16.3	15.1	14.9	12.2	3.80	13.1
	(1) 20.2	**(12)** 14.4	16.3	14.3	13.2	13.3	7.90	15.1
		(13) 14.5	16.3	14.3	13.2	13.3	7.90	13.3
Wk4		**(21)** 15.3	18.7	16.1	14.9	13.7	9.40	14.7
15.5	**(2)** 16.2	**(22)** 17.4	16.7	13.9	14.8	13.3	8.80	14.2
		(23) 16.6	19.6	18.3	13.9	12.9	5.20	14.4
		(31) 16.6	19.6	18.3	13.9	12.9	5.20	14.4
	(3) 18.1	**(32)** 14.4	19.9	17.4	13.6	15.9	13.4	15.8
	LSD 7.97	**(33)** 14.7	9.80	12.6	16.3	13.8	11.9	13.2
	Overall means	15.8	17.2	15.5	14.4	13.5	9.40	
				LSD 3.19				

LSD 4.34

The appropriate LSD values were used to compare differences among the three eight week culture values, and among the nine culture and six subculture values from twelve to thirty-two weeks. These values were obtained from an analysis of variance the results of which are presented in Table 8 of the Appendix. The results of the comparative t-test between values from four to twelve weeks are shown in Table 10 of the Appendix.

Table 3.1.16

CM values for total protein content (TPC) (mg g DW^{-1}) of parent and derived suspension cultures (S) at each subculture during a thirty-two week period. Final column presents the overall means for the nine cultures and the final row presents the overall means at the six subculture times. Each result is the mean of three separate measurements. The numbers in brackets represent the culture numbers.

Parent 15.3			**Subculture (Weeks)**						
		Wk12	**16**	**20**	**24**	**28**	**32**	**Overall Means**	
	Wk8	**(11)** 17.6	13.7	14.3	16.9	23.9	0.00	14.4	
	(1) 14.0	**(12)** 18.8	14.3	17.5	15.7	23.8	10.1	16.7	
		(13) 19.4	11.4	11.3	11.8	23.5	12.0	14.9	
Wk4		**(21)** 17.9	12.7	13.4	13.2	11.3	6.80	12.6	
14.8	**(2)** 17.0	**(22)** 18.2	11.3	13.3	16.3	25.1	7.80	15.3	**LSD 7.06**
		(31) 19.5	14.5	13.4	13.3	7.70	10.9	13.2	
		(31) 18.3	16.4	12.0	14.6	21.6	4.30	14.6	
	(3) 15.1	**(32)** 17.5	6.40	4.70	4.60	11.5	0.00	7.50	
	LSD 7.94	**(33)** 19.5	7.70	0.00	4.20	10.9	0.00	7.10	
	Overall means	18.5	12.0	11.1	12.3	17.8	5.60		
				LSD 5.18					

The appropriate LSD values were used to compare differences among the three eight week culture values, and among the nine culture and six subculture values from twelve to thirty-two weeks. These were obtained from an analysis of variance the results of which are presented in Table 8 of the Appendix. The results of the comparative t-test between values from four to twelve weeks are shown in Table 10 of the Appendix.

The differences among the nine culture lines were due to the significantly lower TPC in cultures IS32 and IS33 particularly at twenty weeks (Table 3.1.17). There was also a significant drop in TPC within cultures after the last subculture, however, the fall within the IS32 and IS33 occurred much earlier in the experiment, so much so that no protein could be detected in these two cultures after the subculture at twenty weeks (Table 3.1.17).

d. iv. Comparison of TPC among regimes and summary of results

The CM values for regimes presented in Table 3.1.18 show no significant differences in TPC among regimes after the subcultures at four and eight weeks. Furthermore over the following twelve to thirty-two week period there were no significant differences found in TPC among the three regimes (Table 3.1.18).

To summarise, differences in TPC were not found among or within cultures derived from a single culture and maintained in different regimes until after the third subculture. In this respect only two cultures derived from the eight week old parent suspension culture S3 showed a significant decline in TPC, that is until the very last subculture. This caused significant differences to arise both within and among the suspension and especially the immobilized suspension cultures; however it was not enough to cause any significant differences among regimes.

Table 3.1.17

CM values for total protein content (TPC) (mg g DW^{-1}) of parent and derived immobilized suspension cultures (IS) at each subculture during a thirty-two week period. Final column presents the overall means for the nine cultures and the final row presents the overall means at the six subculture times. Each result is the mean of three separate measurements. The numbers in brackets represent the culture numbers.

Parent 15.3		**Subculture (Weeks)**						
	Wk12	**16**	**20**	**24**	**28**	**32**	**Overall Means**	
Wk8	**(11)** 19.5	20.5	19.9	15.1	14.4	0.00	14.9	
(1) 13.6	**(12)** 17.0	24.5	18.4	24.8	17.6	20.2	20.4	
	(13) 14.0	16.6	15.0	16.9	19.4	17.3	16.4	
	(21) 18.1	15.2	21.8	15.6	11.4	0.00	13.7	
(2) 14.6	**(22)** 22.4	16.1	23.6	21.4	16.8	0.00	15.1	**LSD 8.18**
	(23) 13.7	14.9	18.8	12.6	14.9	0.00	12.5	
	(31) 19.7	12.1	18.1	14.8	13.8	0.00	13.1	
(3) 15.9	**(32)** 7.70	7.74	0.00	0.00	0.00	0.00	2.56	
LSD 7.94	**(33)** 19.4	11.4	6.10	0.00	0.00	0.00	6.20	
Overall means	16.8	16.4	15.8	13.5	12.0	4.20		
			LSD 6.01					

The appropriate LSD values were used to compare differences among the three eight week culture values, and among the nine culture and six subculture values from twelve to thirty-two weeks. These were obtained from an analysis of variance the results of which are presented in Table 8 of the Appendix. The results of the comparative means t-test between values from eight to twelve weeks are shown in Table 10 of the Appendix.

Table 3.1.18

Comparison of the mean total protein content (TPC) (mg g DW^{-1}) among callus, suspension and immobilized suspension cultures at each subculture during a thirty-two week period. Final column presents the overall means for each regime from twelve to thirty-two weeks.

	Subculture (Weeks)								Overall Means
Regime	**Wk4**	**8**	**12**	**16**	**20**	**24**	**28**	**32**	
Callus	15.3	18.3	15.8	17.2	15.5	14.4	13.5	9.40	14.3
Suspension	14.8	15.3	18.5	12.0	11.1	12.3	17.7	5.80	12.9
Immobilized-Suspension		14.6	16.8	16.4	15.8	13.5	12.0	4.20	13.1
LSD values	**3.34**	**3.89**							**1.71**

The appropriate LSD values were obtained by three individual analysis of variance tests shown in Table 8 and 9 of the Appendix and are used to compare the differences among the overall means at four, eight and between twelve and thirty-two weeks.

e. Soluble phenolic compounds (SPCs) in the medium of the cultures

e. i. Among and within callus cultures

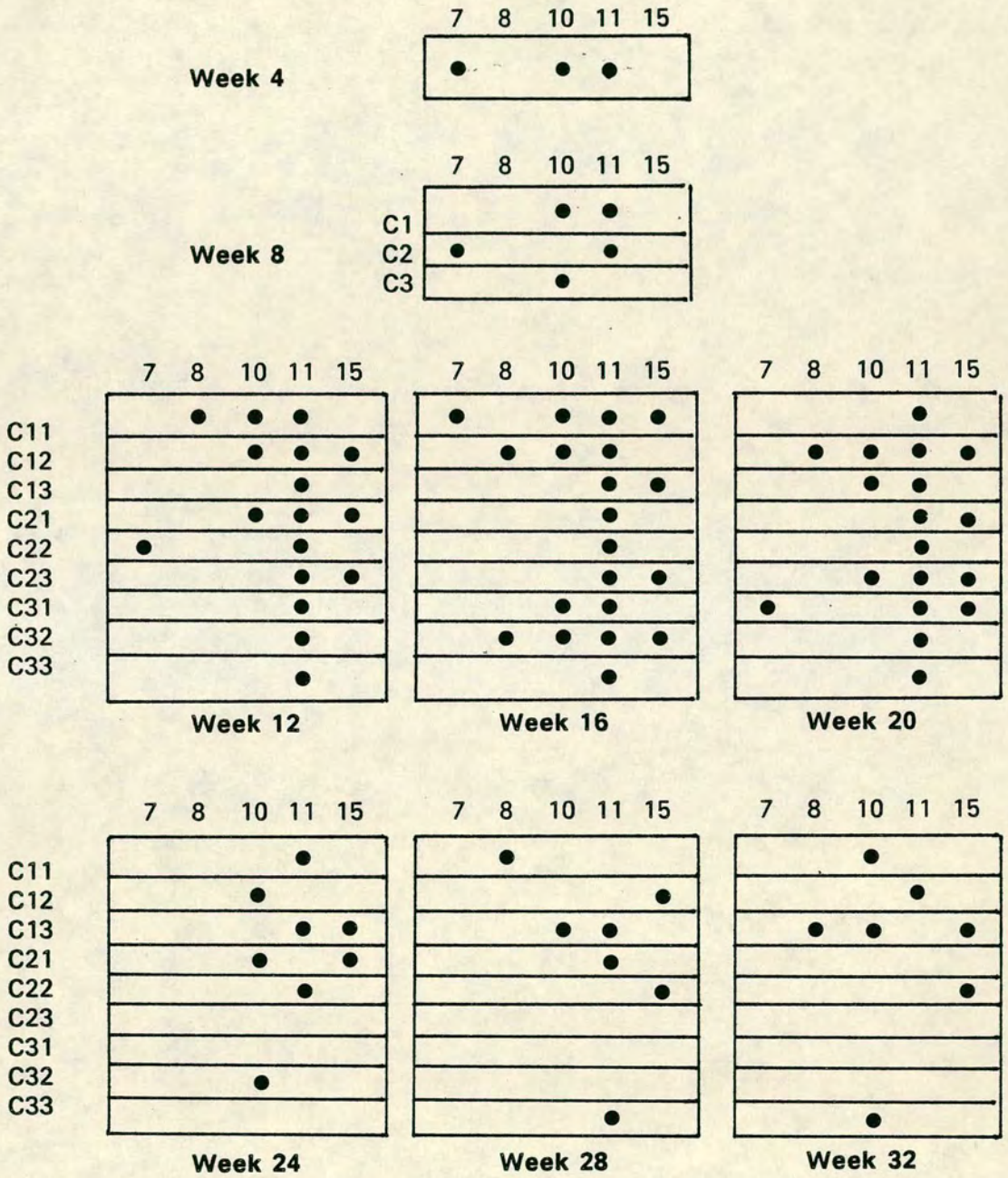
The profiles of the unknown SPCs in the medium of the callus cultures over the thirty-two weeks are shown in 3.1.10. These profiles show that there were no consistent differences among the cultures with regard to the production of these SPCs (Fig 3.1.10). Five SPCs (P7, P8, P10, P11 and P15) were predominant in the medium of the cultures, however P10, P15 and, in particular, P11 were detected to a far greater degree than the other two (Fig 3.1.10). The unknown P11 was produced by all nine cultures at the third, fourth and fifth subcultures, while at the fourth subculture all appeared to be highly active as P10 and P15 were also found to be predominant (Fig 3.1.10). One pattern that emerged from these results was the drop in the number of SPCs within cultures with increasing subculture (Fig 3.1.10). These results also showed that the cultures did not produce SPCs known to be involved in capsaicin synthesis (Fig 3.1.10).

e. ii. Among and within suspension cultures

The profiles of the unknown SPCs in the medium of the suspension cultures over the thirty-two weeks are shown in Fig 3.1.11. Five SPCs (P7, P8, P10, P11 and P12) were present at the first subculture and were predominant over the remainder of the experiment, however there were no consistent differences among the cultures with regard to the appearance of these SPCs (Fig 3.1.11). At the third subculture after twelve weeks, the cultures derived from the eight week old parent culture S2 produced more SPCs than those derived from either S1 or S3 (Fig 3.1.11). However, at the fourth subculture the S1 derived cultures produced as many unknown SPCs as those from S2, infact all nine cultures, apart from S32 and S33 which had stopped producing SPCs, were more active than at any other time during the experiment (Figs 3.1.11, 3.1.13). After the fifth subculture the number and type of SPCs produced by the nine cultures dropped with increasing subculture, although P10 and P11 were noticeable for their presence up to the last subculture (Fig 3.1.11).

Figure 3.1.10

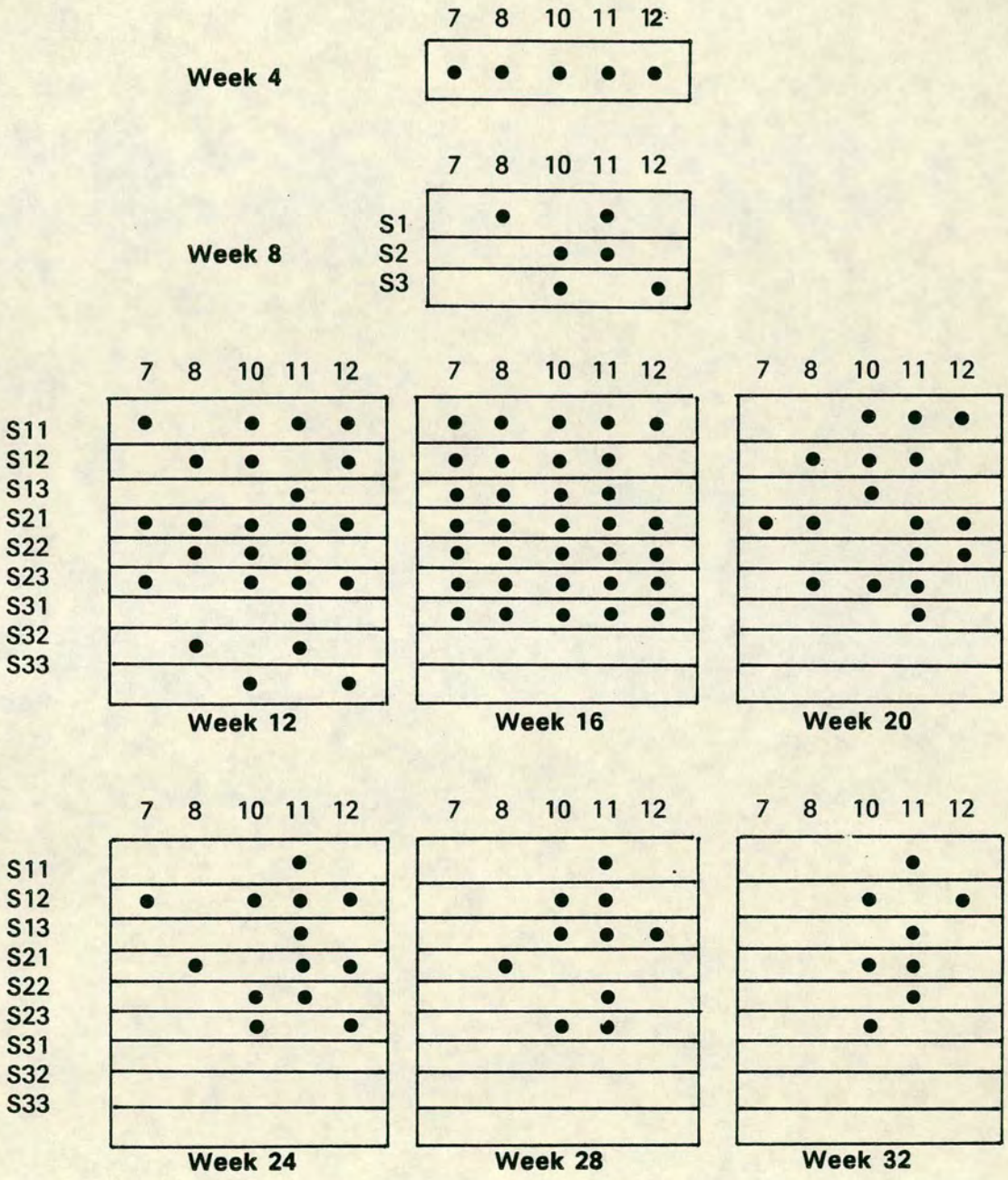
Profiles of unknown $CHCL_3$ soluble phenolic compounds in the medium of callus cultures at each subculture during a thirty-two week period. Each result represents the total of three replicate cultures.



● denotes presence of the phenolic compound

Figure 3.1.11

Profiles of unknown $CHCL_3$ soluble phenolic compounds in the medium of suspension cultures at each subculture during a thirty-two week period. Each result represents the total of three replicate cultures.



It can be seen in Table 3.1.19 that the known SPCs (capsaicin related) were also found to accumulate in the medium of the suspension cultures, although their presence could not be associated with a particular culture. Cinnamic acid was found at the third and, more noticeably, at the fourth subculture after sixteen weeks (Table 3.1.19, Fig 3.1.13). By the fifth subculture vanillin was also found in some and its presence along with cinnamic acid was detected in many cultures even up to the end of the experiment (Table 3.1.19).

e. iii. Among and within immobilized suspension cultures

The profiles of the unknown SPCs found in the medium of the immobilized suspension cultures over the thirty-two weeks are shown in Fig 3.1.12. Five SPCs (P7, P8, P10, P11 and P12) were predominant in the medium of the cultures during the experiment. At the third subculture those derived from the eight week old parent culture S3 appeared to accumulate an appreciably greater number of unknown SPCs (Figs 3.1.12, 3.1.14). However, over the remaining twenty weeks there were no consistent differences among the nine cultures with regard to the production of these unknown SPCs, although there was a drop in the number of SPCs produced with increasing subculture particularly in the medium of cultures IS32 and IS33 (Fig 3.1.12).

It can be seen in Table 3.1.20 that known SPCs (capsaicin related) also accumulated in the medium of the immobilized suspension cultures, however there were no consistent differences found among the cultures with regard to their accumulation. Vanillin, ferulic acid, cinnamic acid and coumaric acid were found in all three cultures after eight weeks, and again in most of the nine cultures at the next subculture after twelve weeks, while cinnamic acid was present in predominantly greater amounts (Table 3.1.20, Fig 3.1.14). At the fourth subculture capsaicin was found in cultures IS21 and IS23, while the cultures IS32 and IS33 were not found to produce any of these five known SPCs (Table 3.1.20). After the fifth subculture there was a drop in both the number and amount of known SPCs produced by the nine cultures so at the last subculture only those derived from the eight week old parent cultures S1 and S2 produced these known SPCs (Table 3.1.20).

Table 3.1.19

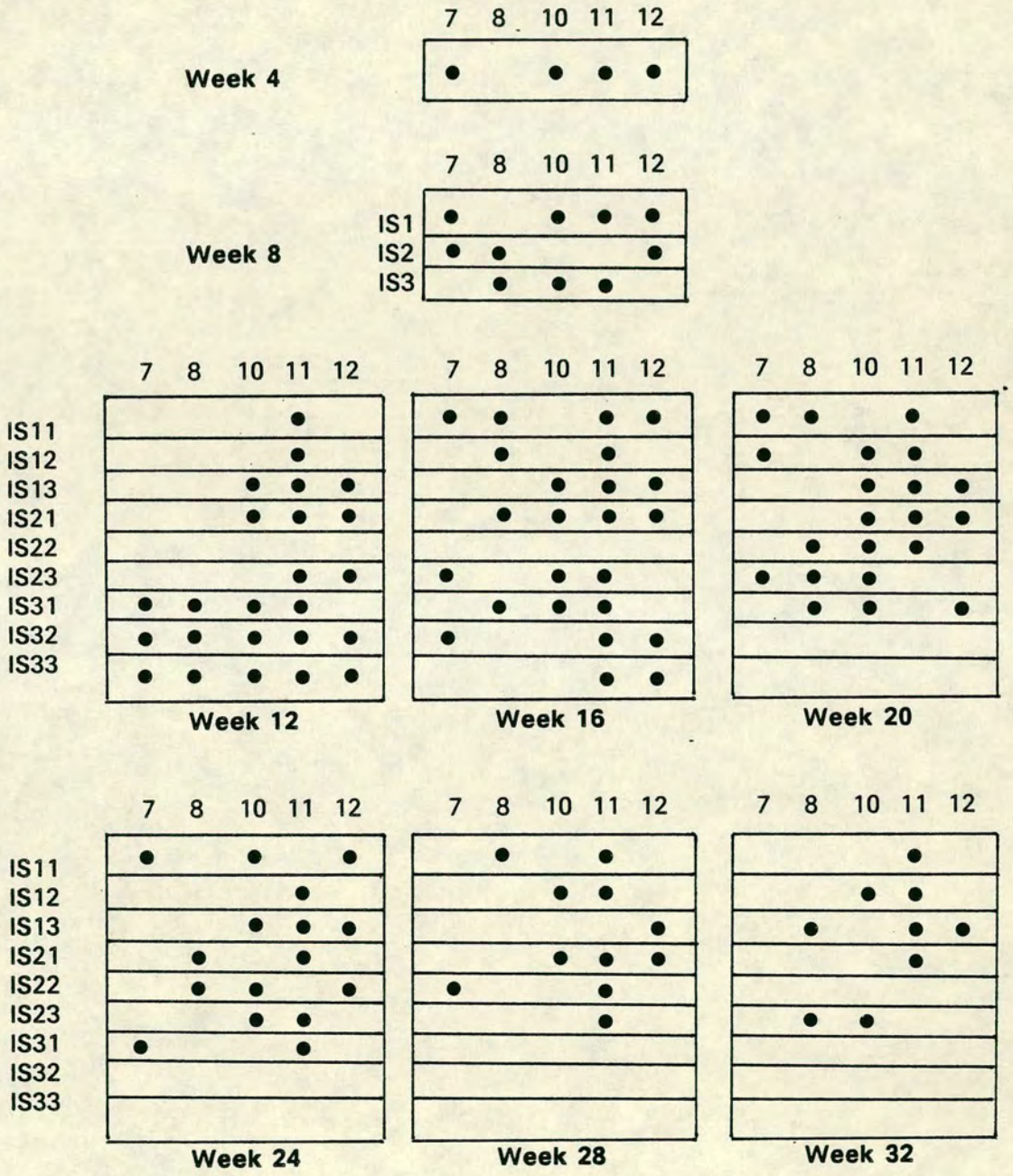
Amounts of known $CHCL_3$ soluble phenolic compounds (µg g DW^{-1}) in the medium of suspension cultures at each subculture during a thirty-two week period. Each result is a mean of three replicate cultures.

		S11	S12	S13	S21	S22	S23	S31	S32	S33
Week 12	CN	2.7	1.5		2.6	5.4	6.3	4.5		
	VN									
Week 16	CN	17.2	9.1	8.5	10.1	7.8	10.3	15.2		
	VN									
Week 20	CN			17.2		16.9		14.1		
	VN			16.5		9.3		8.3		
Week 24	CN	2.9		4.5		12.1				
	VN			17.1	1.9		9.2			
Week 28	CN	5.6	5.2		3.6		2.5			
	VN	10.1	11.3		9.1					
Week 32	CN	17.2	4.5			1.2	14.2			
	VN	3.2	5.1					4.8		

CN = cinnamic acid VN = vanillin

Figure 3.1.12

Profiles of unknown $CHCL_3$ soluble phenolic compounds in the medium of immobilized suspension cultures at each subculture during a thirty-two week period. Each result represents the total of three replicate cultures.



● denotes presence of the phenolic compound

Table 3.1.20

Amounts of known $CHCL_3$ soluble phenolic compounds (µg g DW^{-1}) in the medium of immobilized suspension cultures at each subculture during a thirty-two week period. Each result is a mean of three replicate cultures.

Week 4

	CN	VN	CO	FR	CP
IS		20.0		35.3	

Week 8

	CN	VN	CO	FR	CP
IS1	12.9	48.3	9.4	66.1	
IS2	59.3	17.2	10.1	51.2	
IS3	66.1	9.8	12.1	67.3	

	Week 12					Week 16					Week 20				
	CN	VN	CO	FR	CP	CN	VN	CO	FR	CP	CN	VN	CO	FR	CP
IS11	58.2					67.1	12.0		6.6		7.9	1.1	1.2		
IS12	92.3	65.5		64.0		15.2	28.0	17.2	44.3			1.7	15.6		
IS13	131	42.1		42.8		67.0	17.1	4.0	36.1		37.1	47.8	2.5	12.3	
IS21	111	82.4	32.1	93.4		44.9	31.3	10.1		19.8	23.1	41.2	51.6	21.1	
IS22	81.2					39.2	38.1		17.8		19.6				19.6
IS23	103	83.0		80.0		20.1	91.3		10.1	15.2		1.3	9.7		
IS31	99.5	54.8	18.0	94.1		27.8	17.9		11.2		11.1	31.0		48.1	
IS32	59.2		32.4	123											
IS33	68.7	123		77.9											

	Week 24					Week 28					Week 32				
	CN	VN	CO	FR	CP	CN	VN	CO	FR	CP	CN	VN	CO	FR	CP
IS11						9.6	5.3		3.6						
IS12	12.1	18.3	9.3			11.3	25.7	17.1	29.7		12.1	11.3	3.9	6.0	
IS13		25.1	13.9	5.1		15.3	14.9					41.1	3.8		
IS21	19.1	33.0	5.4	11.1		9.8	23.1	43.7		19.6	15.3	5.1			
IS22		34.0		12.9							17.8		4.0		
IS23	17.2														
IS31															
IS32															
IS33															

CN = cinnamic acid, VN = vanillin, CO = coumaric acid, FR = ferulic acid
CP = capsaicin.

e. iv. Comparison of the SPCs in the medium among regimes and summary of results

Differences were found among regimes with respect to the production of unknown SPCs. Although the cultures in all three regimes produced the same unknown SPCs, suspension and immobilized cultures were notable for producing more of these throughout the experiment. There were more pronounced differences among regimes with regard to the production of known capsaicin related SPCs. Callus cultures did not produce any of these at any time during the course of the experiment, however suspension and notably immobilized suspension cultures did produce these SPCs.

In this respect, suspension cultures produced two known SPCs, cinnamic acid and vanillin after the third subculture while the immobilized suspension cultures produced these two as well as coumaric acid, ferulic acid and capsaicin at different times throughout the whole experiment. Not only were there a greater number of these SPCs in this regime, but the amounts produced were greater in the immobilized suspension than in the suspension cultures.

To summarise, consistent differences in SPCs were not found among or within cultures derived from a single culture until after the fifth subculture at twenty weeks. In this respect there was a drop in SPCs within cultures, notably in the suspension and immobilized suspension cultures derived from the eight week old parent culture S3. The cultures in all three regimes were generally found to produce the same unknown SPCs, of which P11 was the most prominent. However the suspension and especially the immobilized suspension cultures not only produced more of these, but also detectable amounts of known capsaicin pathway intermediates. In general, the production of all SPCs fell with increasing subculture in cultures of all regimes.

Figure 3.1.13

HPLC chromatograms of known and unknown $CHCL_3$ soluble phenolic compounds found in the medium of nine suspension cultures after the fourth subculture at sixteen weeks. The unknown SPCs are represented by their number. The known SPCs are given as Cn= cinnamic acid.

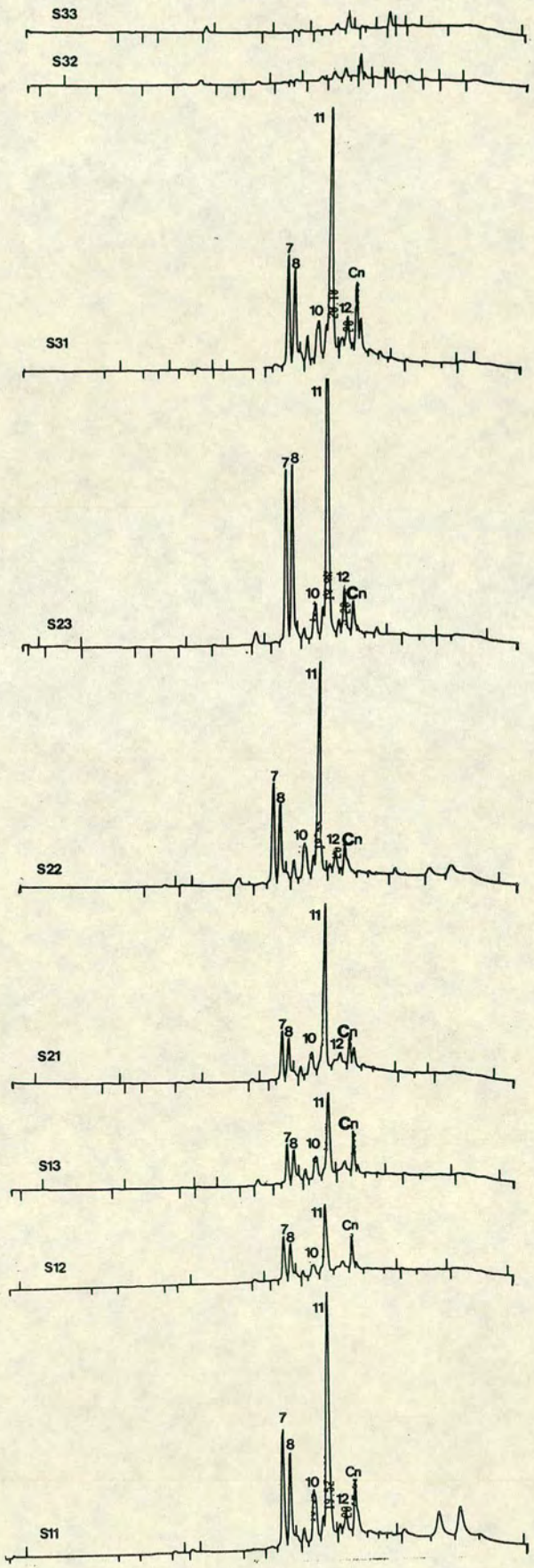
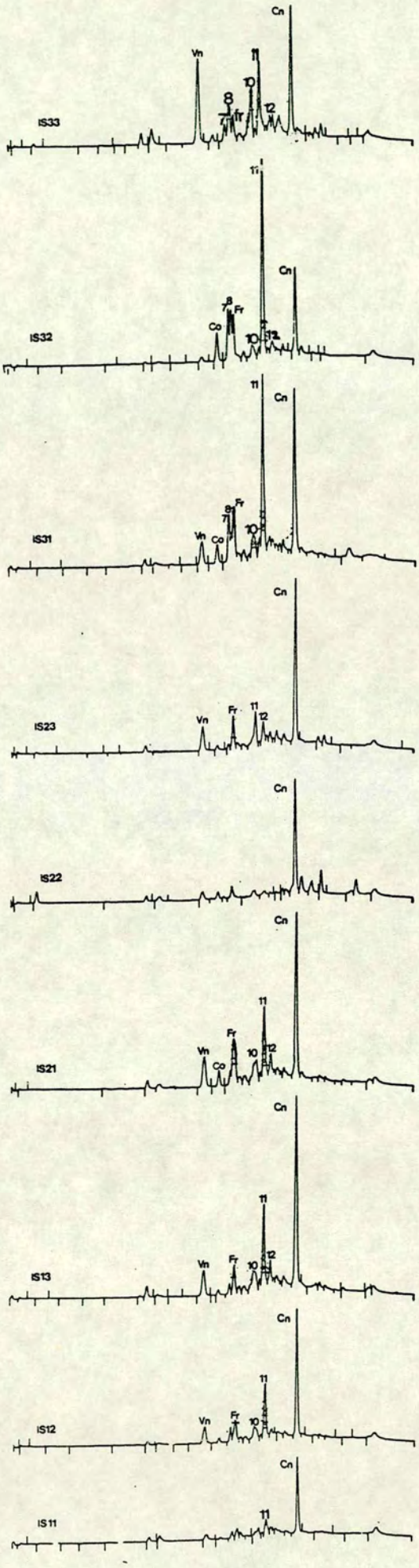


Figure 3.1.14

HPLC chromatograms of known and unknown $CHCL_3$ soluble phenolic compounds in the medium of nine immobilized suspension cultures after the third subculture at twelve weeks. The unknown SPCs are represented by their number. The known SPCs are given as Cn= cinnamic acid, Co= coumaric acid, Fr= ferulic acid and Vn= vanillin.


IS33
Vn
Cn
11
10
8
12
IS32
Co
Fr
IS31
IS23
IS22
IS21
IS13
IS12
IS11

f. Summary of all results of comparisons among and within cultures derived by subculture of a clone over a thirty-two week period

There were no clear differences in the morphological and metabolic characteristics among daughter cultures derived from a single callus culture in callus, suspension or immobilized suspension over the first twelve weeks of the experiment. Any that did occur among the cultures were restricted to the production of SPCs however these differences were sporadic and inconsistent. The only outstanding difference that occurred over this time was a significant drop in the TCC within all cultures in each regime.

After the third subculture, at twelve weeks, differences among and within the cultures started to appear. However, cultures maintained as callus exhibited the least of these differences. Although becoming increasingly variegated the nine callus cultures remained predominantly yellow and friable and maintained a high and stable RGR, TCC and TPC. Differences in SPCs were noticeable although inconsistent, however, there was a drop in the number with increasing subculture.

On the other hand, cultures maintained as suspensions or in the immobilized state had significantly lower RGR, TCC and TPC values and exhibited marked differences after the third subculture which arose from the three suspension cultures derived from the first subculture. It was found that although all cultures became increasingly variegated there were some differences in aggregation and browning, TCC and SPCs associated with the daughter cultures derived from each of these three cultures. In particular one set of cultures showed a sharp decline in RGR, TCC, TPC and SPCs while browning and aggregation increased and this restricted proper immobilization. Not suprisingly these results appeared to account for the differences found among all nine suspension and immobilized suspension cultures and also between the cultures of these two regimes and callus. These differences could therefore be attributed to those existing within the original parent culture, however it is more likely that the culture regime had a greater effect in causing these to arise and that subculturing separated them into specific culture groups.

Differences in SPCs were also noticeable among and within cultures in both the suspension and immobilized suspension regimes, however these were inconsistent and could not be related to a specific subculture. However, the cultures which showed increased browning and aggregation also exhibited a rapid drop in the number of SPCs. Nevertheless, the other cultures in these two regimes were noticeably more active than callus cultures as they produced many known capsaicin pathway intermediates particularly in the immobilized regime.

PART TWO

THE EFFECT OF MEDIUM CONSTITUENTS ON MORPHOLOGICAL AND METABOLIC CHARACTERISTICS OF SUSPENSION CULTURES

3.2 The effect of medium constituents on morphological and metabolic characteristics of suspension cultures

There are reports which describe the effects of constituents of the medium on changes in the morphological and metabolic processes of plant cell cultures (Mantell and Smith 1983). It has been found that altering the concentration of some of these components can modify essential plant cell processess such as growth and secondary metabolism.

The following section consists of five experiments which were carried out to determine the nature and degree of differences in cultures exposed to different concentrations of five essential medium constituents.

3.2.1 Experimental procedure (see Fig 3.2.1)

A highly dispersed yellow-green suspension culture of clone AE2 with a high RGR, from a previous series of experiments was used. It was randomised and subcultured into five suspension cultures all maintained in SH medium. Each of these was designated the parent culture and provided source material for each of the five treatments namely; 2,4-D, sucrose, nitrate, phosphate and pH (Table 3.2.1). Subcultures were then carried out four and eight weeks later providing enough material for replication for the five 'concentrations' within each treatment (Table 3.2.1).

Each suspension culture was maintained for sixteen weeks during which a subculture was made every four weeks. A determination of appearance, relative growth rate (RGR), total chlorophyll content (TCC) and phenylammonia lyase activity (PAL) was made using the cells remaining after each subculture. All the data obtained at each subculture with the exception of appearance were subjected to an analysis of variance.

Figure 3.2.1

Diagram showing the origins and relationships of experimental material in multiplication phase and the timing of data collection within the treatment phase.

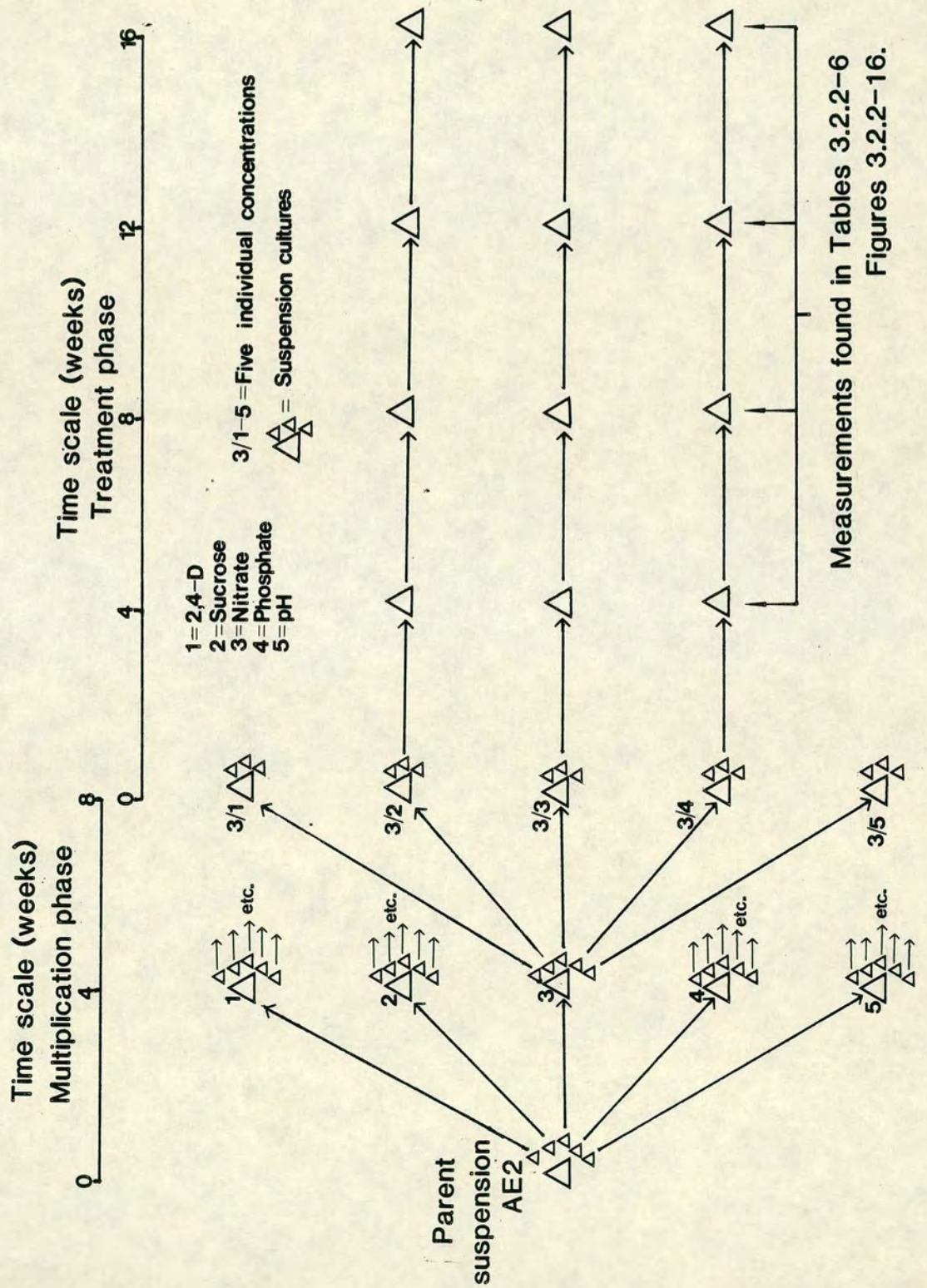


Table 3.2.1

The concentration (mg l^{-1}) of medium constituents and pH used to study the effect of the environment on the morphological and metabolic characteristics of suspension cultures. Three replicates were used for each treatment.

Treatment	Concentration				
2,4-D	0	0.25	0.50 *	0.75	1.0
Sucrose	0	15	30 *	45	60
Nitrate	0	100	300 *	500	700
Phosphate	0	20	40 *	60	80
pH	4.8	5.4	5.8 *	6.2	6.6

* denotes the amount used in standard SH medium

3.2.2 The effect of 2,4-D on the morphological and metabolic characteristics of suspension cultures

3.2.2.1 Appearance of cultures

It can be seen from Table 3.2.2 that the appearance of most of the suspension cultures changed when grown at different 2,4-D concentrations, only the cultures in the standard concentration remained unchanged during the sixteen weeks. Although cells maintained at the two highest 2,4-D concentrations remained mostly yellow and friable with green or white cells, those at the lowest concentration, or in medium without 2,4-D became green and aggregated with increasing subculture (Table 3.2.2).

3.2.2.2 Relative growth rate (RGR) of cultures

The results presented in Figs 3.2.2 a. show that cells maintained at the two highest 2,4-D concentrations had a significantly lower RGR than the other treatments. However, these differences were a result only of the low RGR of cultures in 0.75 mg l^{-1} 2,4-D at four weeks and in 1.0 mg l^{-1} 2,4-D at four and eight weeks, as all RGR values were similar over the latter part of the experiment (Fig 3.2.2 b). The high RGR of cultures maintained in medium without 2,4-D indicated that these cultures were capable of auxin independent growth (Fig 3.2.2 a).

In Fig 3.2.2 c. it can be seen that there was a significantly higher RGR in cultures at the third subculture after twelve weeks. However, the significance of this high value could be explained by the low RGR of cultures at the two highest 2,4-D concentrations over the first eight weeks (Fig 3.2.2 d).

3.2.2.3 Total chlorophyll content (TCC) of cultures

The results presented in Fig 3.2.3. a show that the TCC was significantly lower in cultures maintained at the two highest 2,4-D concentrations (Fig 3.2.3 a). These differences were attributed to the high TCC of cultures at 0.25 mg l^{-1}, 0.50 mg l^{-1} and in medium without 2,4-D at the third and fourth subculture only, and not over the first eight weeks where all the TCC values were similar (Fig 3.2.3 b).

Table 3.2.2

The effect of 2,4-D concentration (mg l^{-1}) on the appearance of suspension cultures over sixteen weeks. Each score is a mean of three sets of observations.

Parent Y(G),F

		Subculture (Weeks)			
	2,4-D Conc.	**Week 4**	**Week 8**	**Week 12**	**Week 16**
	0	Y(G),A	G(Y),A	G,A	G,A
	0.25	Y(G),A	G,A	G,A(F)	G,A(F)
*	0.50	Y(G),F	Y(G),Fr	Y(G),F	Y(G),F(A)
	0.75	Y(G),F	Y(G),F	Y(V),F	Y(V),F
	1.0	Y,F	Y(G),F	Y(V),F	Y(V),F

* denotes the concentration used in standard SH medium

Y=Yellow
G=Green
B=Brown
W=White
F=Friable
A=Aggregated
V=Variegated

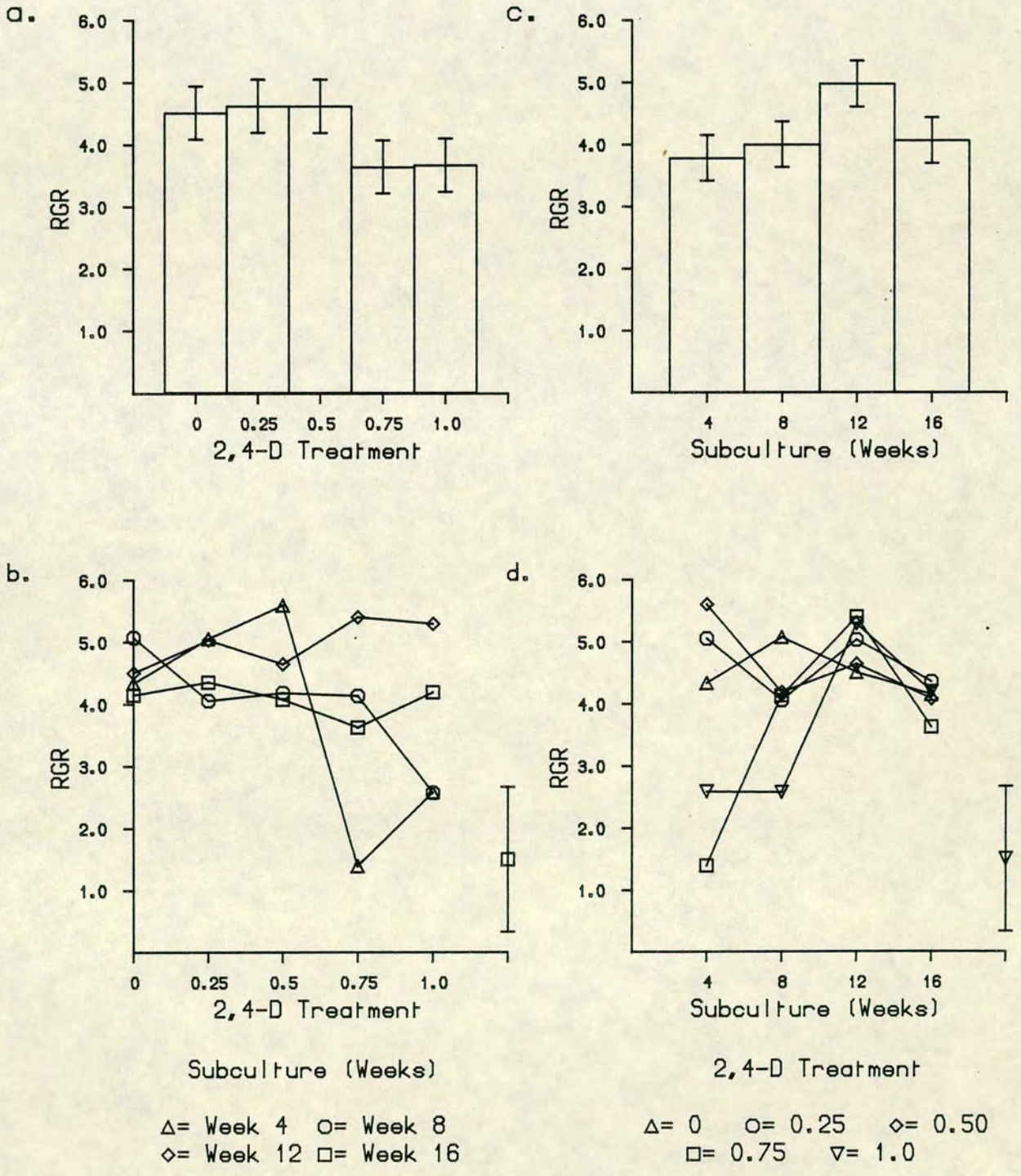
Y(G) means culture mainly yellow with the presence of a small amount of cells of one other colour, in this case green.

Y(V) means culture mainly yellow with the presence of small amounts of other coloured cells (in this case white, green or brown) giving the culture a variegated appearance.

F(A) means culture mostly friable with small amounts of aggregated cells.

Figure 3.2.2

The effect of 2,4-D concentration (mg l^{-1}) and subculture on the relative growth rate (RGR) ($day^{-1}x10^{-2}$) of suspension cultures over sixteen weeks. The histograms a,c show the overall effects of treatment and subculture respectively, and the line graphs b,d show the individual effects of subculture at each concentration, and concentration at each subculture time respectively. Error bars represent the LSD values which were determined from the analysis of variance shown in Table 11 of the Appendix.



In Fig 3.2.3 c. it can be seen that there was a significantly higher TCC in cultures after each of the last two subcultures; however, this increase was not observed in cultures at either of the two highest 2,4-D concentrations especially those growing at 0.75 mg l^{-1} (Fig 3.2.3 d).

3.2.2.4 PAL activity of cultures

The results presented in Fig 3.2.4 a. show that cultures maintained at the highest 2,4-D concentration had a significantly greater PAL activity than the others. This response to different concentrations was noticeable particulary at the second subculture after eight weeks, although there were no differences in PAL activity after the last subculture (Fig 3.2.4 b).

In Fig 3.2.4 c. it can be seen that there was a significant drop in PAL activity in the cultures at the last subculture after sixteen weeks. This response to subculture was found in all the cultures although more markedly in those growing at the highest concentration of 2,4-D (Fig 3.2.4 d).

3.2.2.5 Summary of the effect of 2,4-D on the RGR, TCC and PAL activity of suspension cultures

Varying the 2,4-D concentration caused marked changes in the appearance, RGR, TCC and PAL activity of the suspension cultures over the sixteen weeks. Differences in appearance were most notable at low 2,4-D as cultures growing at the lowest concentration or in medium without 2,4-D turned green and aggregated with increasing subculture. These changes coincided with an increase in TCC over the last two subcultures while these cultures exhibited a high RGR throughout the experiment. Infact, the high RGR of cultures in medium without 2,4-D showed that these cultures are capable of auxin independent growth. At the two highest 2,4-D concentrations, cultures remained yellow and friable with a low RGR which increased rapidly with subculture and a low TCC throughout the experiment. The appearance of cultures growing in the standard 2,4-D concentration changed little over the sixteen weeks while the RGR remained high and the TCC increased with subculture. A high PAL activity was found in cultures at the highest 2,4-D concentration until twelve weeks after which there was a sharp decline in activity in these as well as all the other cultures.

Figure 3.2.3

The effect of 2,4-D concentration (mg l^{-1}) and subculture on the total chlorophyll content (TCC) (µg g DW^{-1}) of suspension cultures over sixteen weeks. The histograms a,c show the overall effects of treatment and subculture respectively, and the line graphs b,d show the individual effects of subculture at each concentration, and concentration at each subculture time respectively. Error bars represent the LSD values which were determined from the analysis of variance shown in Table 11 of the Appendix.

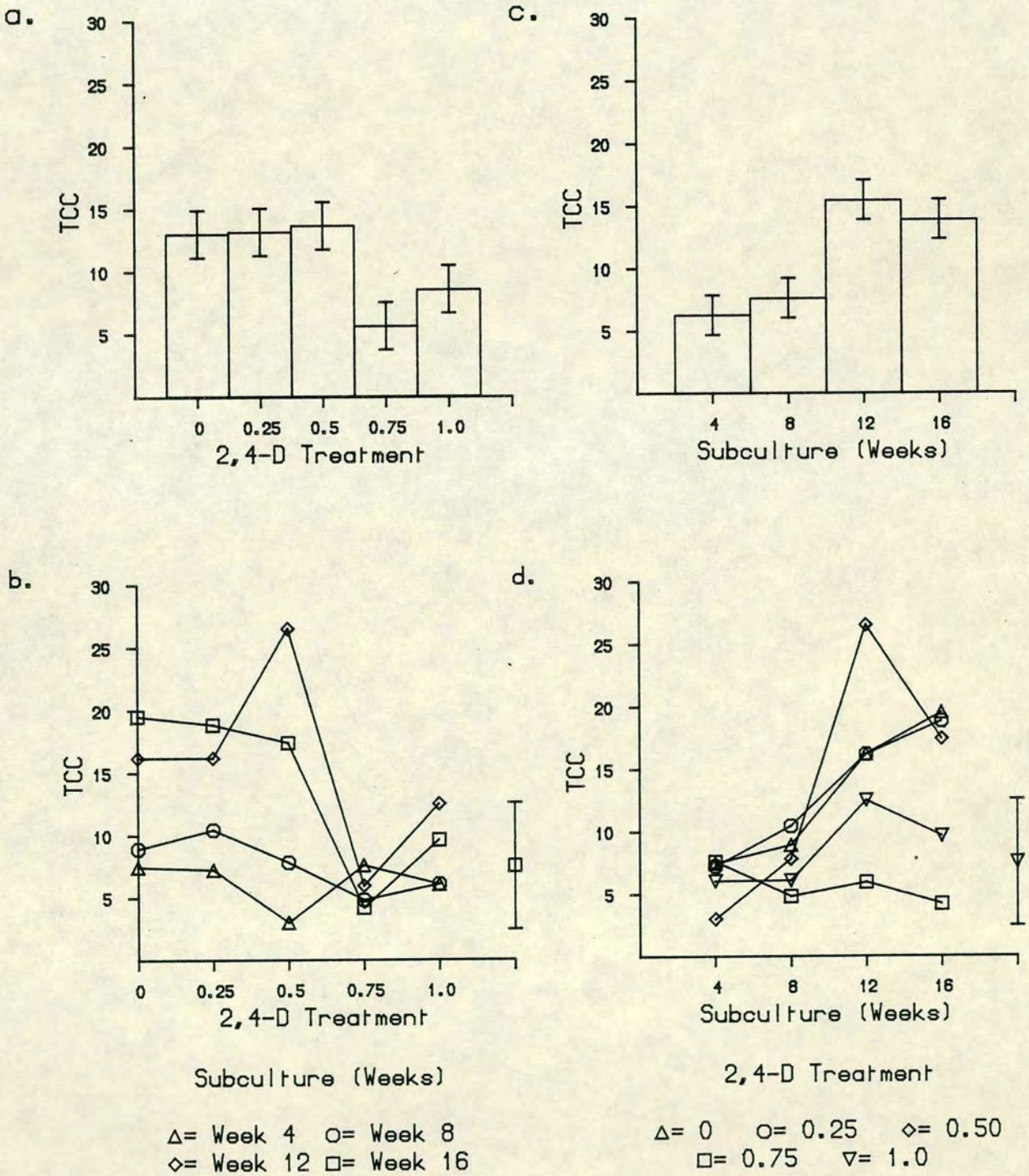
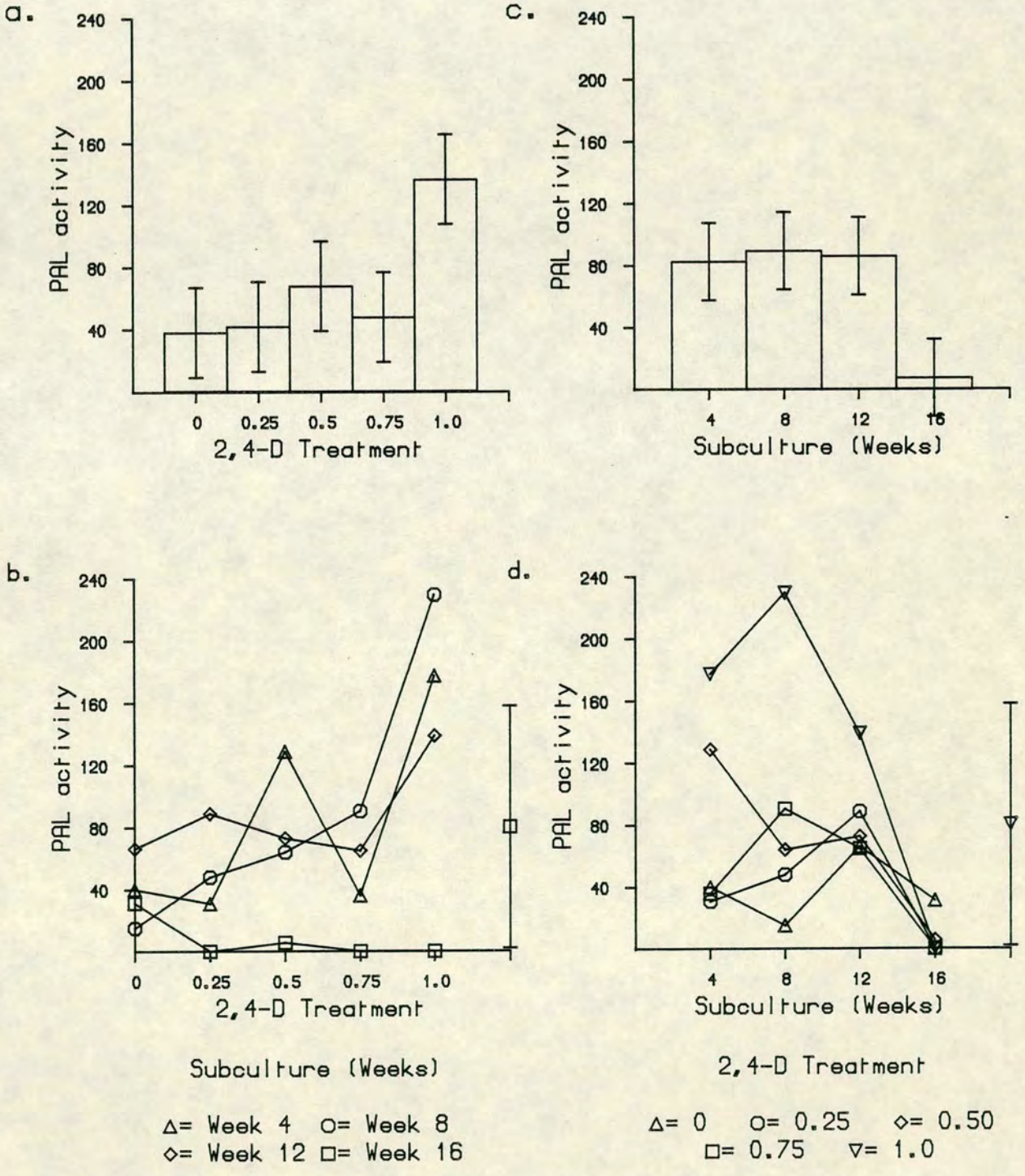


Figure 3.2.4

The effect of 2,4-D concentration (mg l^{-1}) and subculture on the phenylalanine ammonia lyase activity (PAL) (µkat kg^{-1}) of suspension cultures over sixteen weeks. The histograms a,c show the overall effects of treatment and subculture respectively, and the line graphs b,d show the individual effects of subculture at each concentration, and concentration at each subculture time respectively. Error bars represent the LSD values which were determined from analysis of variance shown in Table 11 of the Appendix.



3.2.3 The effect of sucrose on the morphological and metabolic characteristics of suspension cultures

3.2.3.1 Appearance of cultures

It can be seen from Table 3.2.3 that there was a change in the appearance of suspension cultures grown at different sucrose concentrations. Although cells maintained in the two highest sucrose concentrations remained mainly yellow and friable with some green cells, those in the lowest concentration or in medium without sucrose became increasingly brown and aggregated with subculture (Table 3.2.3). Cells growing in the standard concentration although remaining mainly yellow and friable turned green after the third subculture (Table 3.2.3).

3.2.3.2 Relative growth rate (RGR) of cultures

The results presented in Figs 3.2.5 a. show that cells maintained at the lowest concentration, or especially in medium without sucrose had a significantly lower RGR than the other treatments. However, cells growing in the two highest sucrose concentrations also had a low RGR at the first subculture after four weeks (Fig 3.2.5 b).

In Fig 3.2.5 c. it can be seen that there was a significantly greater RGR in cultures at the second and third subcultures over the sixteen week period. However this pattern of response was not observed in cells growing in medium without sucrose because growth had stopped in these cultures after the first subculture at four weeks (Fig 3.2.5 d).

3.2.3.3 Total chlorophyll content (TCC) of cultures

The results presented in Fig 3.2.6 a. show that cells maintained at the standard sucrose concentration had a significantly greater TCC than cells in the lowest sucrose concentration, or in medium without sucrose. These results also show that chlorophyll was not found in cultures growing in medium without sucrose making this significantly lower than all other cultures (Fig 3.2.6 a). This pattern of response to sucrose concentration was attributed mainly to the high TCC in cultures at the standard concentration and the low TCC in cultures at the two highest concentrations at the third subculture (Fig 3.2.6 b).

Table 3.2.3

The effect of sucrose concentration (mg l^{-1}) on the appearance of suspension cultures over sixteen weeks. Each score is a mean of three sets of observations.

Parent Y(G),F

		Subculture (Weeks)			
	Sucrose Conc.	**Week 4**	**Week 8**	**Week 12**	**Week 16**
	0	Y,A	B,A	B,A	B,A
	15.0	Y(G),F(A)	B(G),A	B(G),A	B(G),A
*	30.0	Y(G),F	Y(G),F	G,F	G,F
	45.0	Y,A	Y(G),F(A)	Y,F(A)	Y(G),F
	1.0	Y,F	Y(G),F	Y(G),F	G(Y),F

* denotes the concentration used in standard SH medium

Y=Yellow

G=Green

B=Brown

W=White

F=Friable

A=Aggregated

V=Variegated

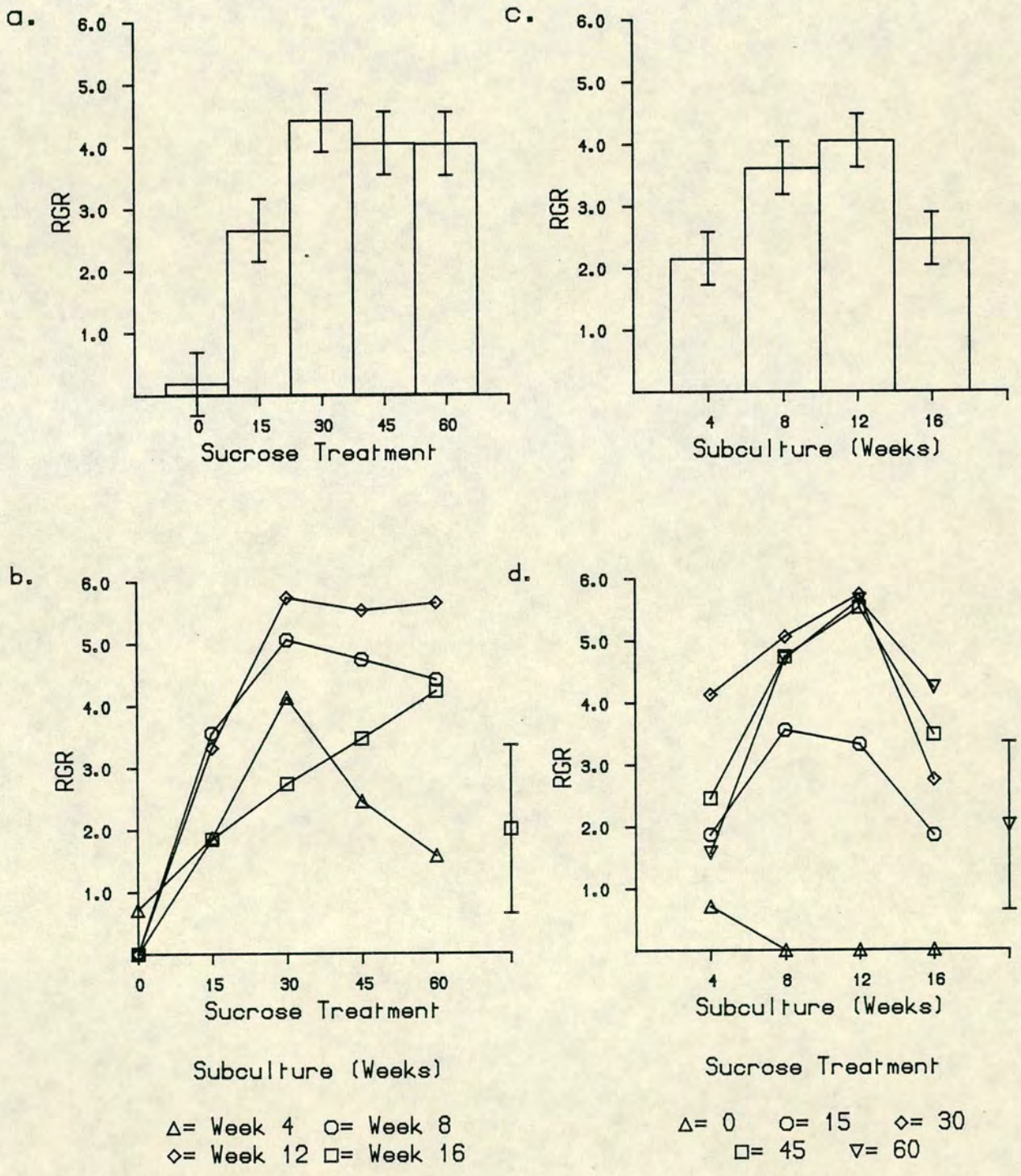
Y(G) means culture mainly yellow with the presence of a small amount of cells of one other colour, in this case green.

Y(V) means culture mainly yellow with the presence of small amounts of other coloured cells (in this case white, green or brown) giving the culture a variegated appearance.

F(A) means culture mostly friable with small amounts of aggregated cells.

Figure 3.2.5

The effect of sucrose concentration (mg l^{-1}) and subculture on the relative growth rate (RGR) ($day^{-1}x10^{-2}$) of suspension cultures over sixteen weeks. The histograms a,c show the overall effects of treatment and subculture respectively, and the line graphs b,d show the individual effects of subculture at each concentration, and concentration at each subculture time respectively. Error bars represent the LSD values which were determined from the analysis of variance shown in Table 12 of the Appendix.



In Fig 3.2.6 c. it can be seen that although there was an increase in TCC with subculture there was only a significant difference between the first and the last measurements. However, this response was not observed in cells growing at the lowest or standard sucrose concentration after twelve weeks as the TCC dropped, or even in cells growing in medium without sucrose when chlorophyll could not be detected at any time in the experiment (Figs 3.2.6 d).

3.2.3.4 PAL activity of cultures

The results presented in Figs 3.2.7 a. and b. show that cells growing in all the sucrose concentrations had a low and insignificant PAL activity and that this response was observed after each subculture of the experiment. It was also seen in Figs 3.2.7 c. and d. that the PAL activity of the cultures was not affected by subculture in any of the sucrose concentrations during the sixteen weeks.

3.2.3.5 Summary of the effect of sucrose concentration on the RGR, TCC and the PAL activity of the suspension cultures

Varying the sucrose concentration caused marked changes in the appearance, RGR and TCC of the suspension cultures over the sixteen weeks. Differences in appearance were most notable in cultures growing at the lowest concentration, or in medium without sucrose. These cultures became increasingly brown and aggregated with subculture which coincided with a significantly lower TCC and RGR; infact cultures in medium without sucrose stopped growing after the first subculture and did not produce chlorophyll at any time during the sixteen weeks. Cultures growing at the two highest sucrose concentrations remained mostly yellow and friable with some occasional greening over the subcultures. These observations were accompanied by a high RGR after eight and twelve weeks and a TCC which which was high by the last subculture. The colour of cultures growing at the standard sucrose concentration changed from yellow to green over the latter half of the experiment. This change coincided with an increase in TCC and a high RGR which dropped after the final subculture. The PAL activity was low throughout the experiment and did not respond to any changes in sucrose concentration.

Figure 3.2.6

The effect of sucrose concentration (mg l^{-1}) and subculture on the total chlorophyll content (TCC) (µg g DW^{-1}) of suspension cultures over sixteen weeks. The histograms a,c show the overall effects of treatment and subculture respectively, and the line graphs b,d show the individual effects of subculture at each concentration, and concentration at each subculture time respectively. Error bars represent the LSD values which were determined from the analysis of variance shown in Table 12 of the Appendix.

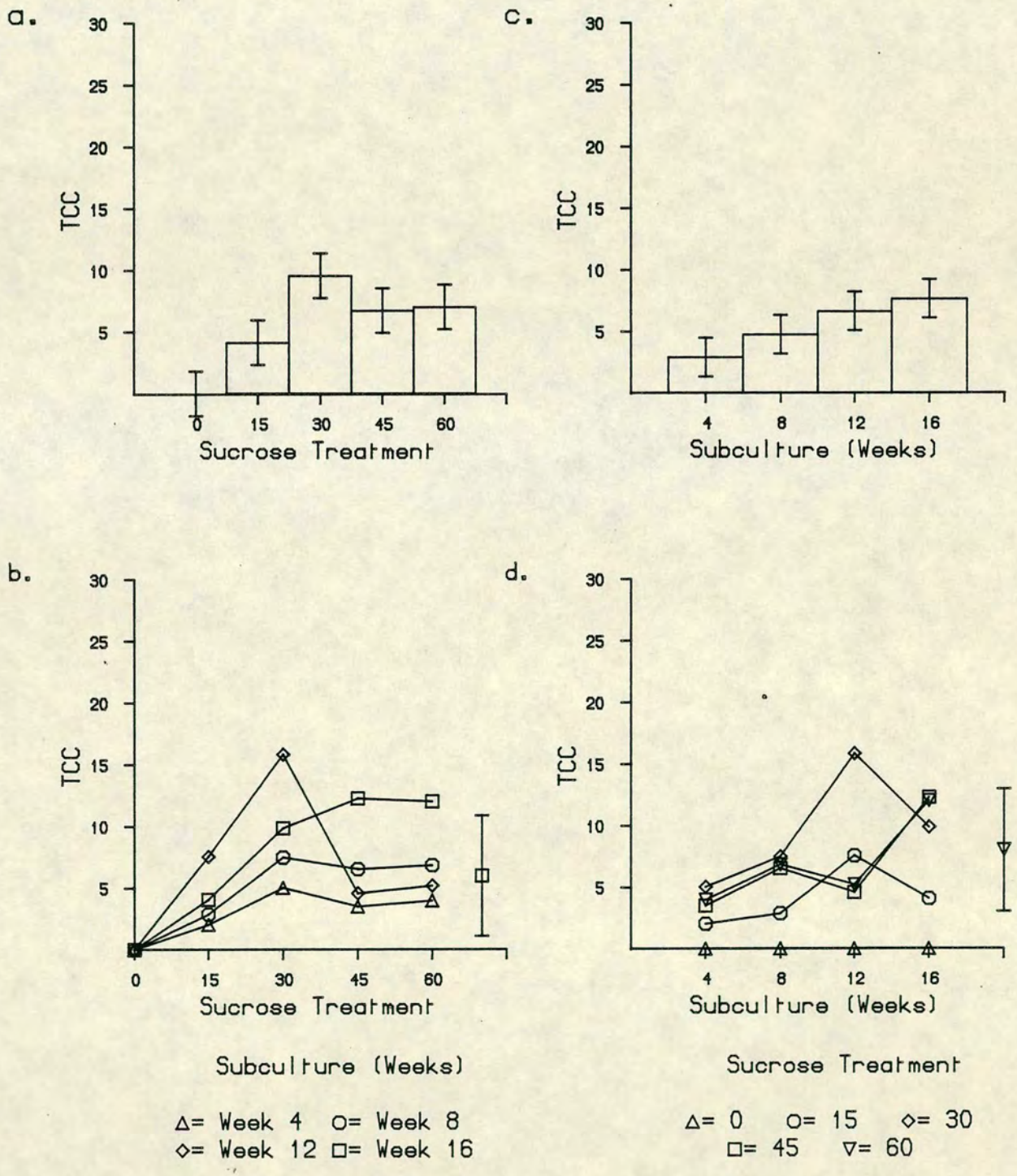
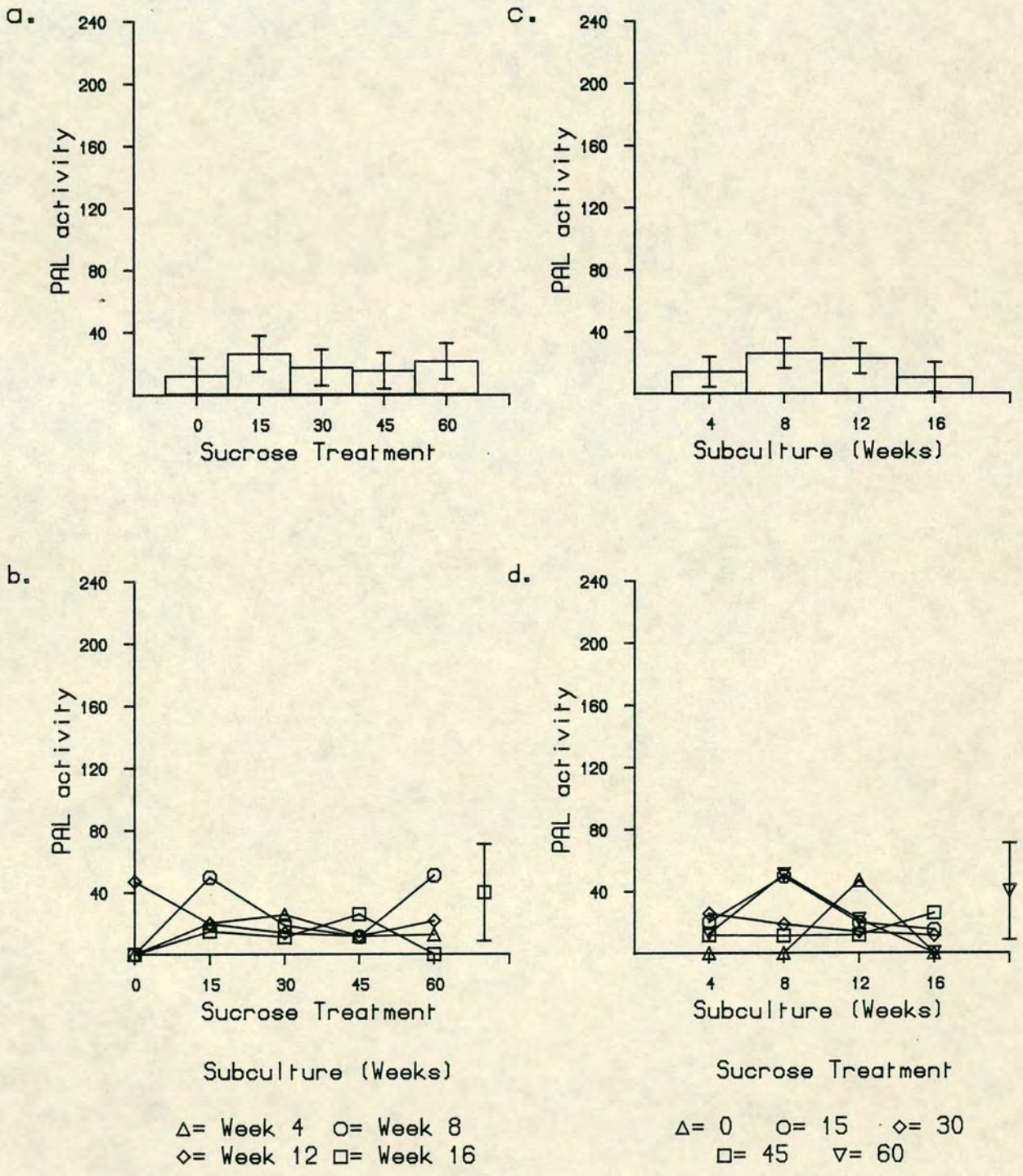


Figure 3.2.7

The effect of sucrose concentration (mg l^{-1}) and subculture on the phenylalanine ammonia lyase activity (PAL) (µkat kg^{-1}) of suspension cultures over sixteen weeks. The histograms a,c show the overall effects of treatment and subculture respectively, and the line graphs b,d show the individual effects of subculture at each concentration, and concentration at each subculture time respectively. Error bars represent the LSD values which were determined from analysis of variance shown in Table 12 of the Appendix.



3.2.4 The effect of nitrate on the morphological and metabolic characteristics of suspension cultures

3.2.4.1 Appearance of cultures

It can be seen from Table 3.2.4 that there was little change in the appearance of the suspension cultures when grown in different nitrate concentrations. However, cells maintained in medium without nitrate or, to a lesser extent, at the lowest nitrate concentration, became brown and aggregated with increasing subculture, and those in the highest nitrate concentration became visibly greener near the end of the sixteen weeks.

3.2.4.2 Relative growth rate (RGR) of cultures

The results presented in Figs 3.2.8 a. show that there was a significant increase in RGR with increasing nitrate concentration, and cultures with the greatest RGR were found at the two highest concentrations. This response to nitrate was found after each subculture especially at eight, twelve and sixteen weeks where the values for RGR within each concentration were very similar (Fig 3.2.8 b).

In Fig 3.2.8 c. it can be seen that there was a significantly higher RGR in cultures at the first subculture after four weeks, a response that was found at every nitrate concentration. However, the reduction in growth of cells in medium without nitrate after four weeks was so pronounced that the cells stopped growing (Fig 3.2.8 d).

3.2.4.3 Total chlorophyll content (TCC) of cultures

The results presented in Fig 3.2.9 a. show that cells maintained at the two highest nitrate concentrations, and especially at 700 mg l^{-1}, accumulated significantly higher amounts of chlorophyll than the others. This response to high nitrate concentration was attributed mainly to the very high TCC of cultures at both these concentrations after the last two subcultures (Fig 3.2.9 b.).

Table 3.2.4

The effect of nitrate concentration (mg l^{-1}) on the appearance of suspension cultures over sixteen weeks. Each score is a mean of three sets of observations.

Parent Y(G),F

		Subculture (Weeks)			
	Nitrate Conc.	**Week 4**	**Week 8**	**Week 12**	**Week 16**
	0	Y,(F)A	Y(B),A	B(Y),A	B,A
	100	Y(G),F	Y(B),F	Y(B),F(A)	Y(B),A
*	300	Y(G),F	Y(G),F	Y(G),F(A)	Y(G),F(A)
	500	Y(G),F	Y(G),F	Y(G),F	Y(G),F(A)
	700	Y(G),F	Y(G),F	G(Y),F	G(Y),F

* denotes the concentration used in standard SH medium

Y=Yellow
G=Green
B=Brown
W=White
F=Friable
A=Aggregated
V=Variegated

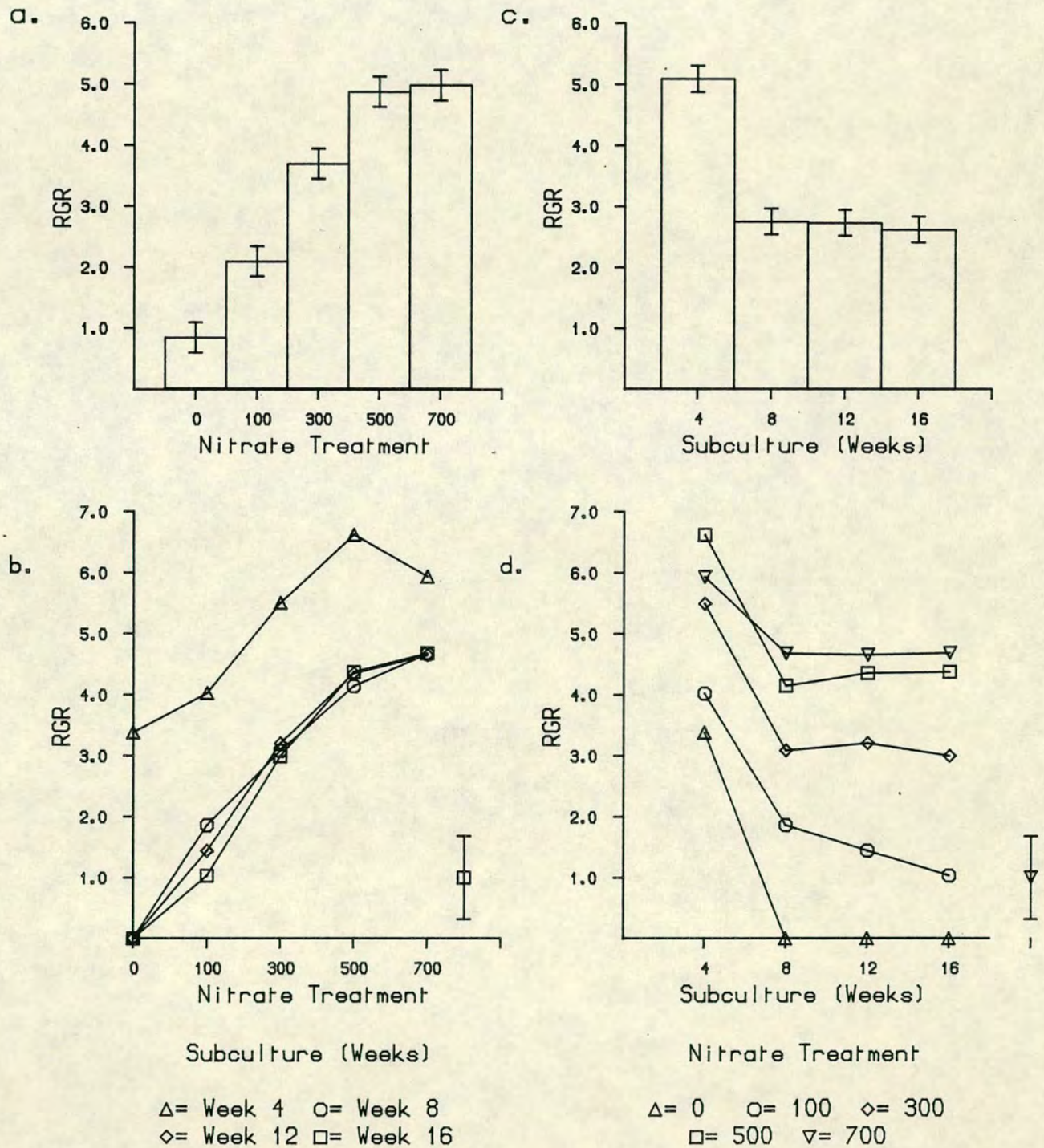
Y(G) means culture mainly yellow with the presence of a small amount of cells of one other colour, in this case green.

Y(V) means culture mainly yellow with the presence of small amounts of other coloured cells (in this case white, green or brown) giving the culture a variegated appearance.

F(A) means culture mostly friable with small amounts of aggregated cells.

Figure 3.2.8

The effect of nitrate concentration (mg l^{-1}) and subculture on the relative growth rate (RGR) ($day^{-1}x10^{-2}$) of suspension cultures over sixteen weeks. The histograms a,c show the overall effects of treatment and subculture respectively, and the line graphs b,d show the individual effects of subculture at each concentration, and concentration at each subculture time respectively. Error bars represent the LSD values which were determined from the analysis of variance shown in Table 13 of the Appendix.



In Fig 3.2.9 c. it can be seen that there were significantly higher amounts of chlorophyll in cultures after each of the last two subcultures, however, the significance of the TCC at these times was attributed solely to the accumulation in cultures at the two highest concentrations (Fig 3.2.9 d). In fact there was no chlorophyll found in cultures growing at the lowest concentration or in medium without nitrate after four weeks, or in the standard concentration after eight weeks (Figs 3.2.9 d).

3.2.4.4 PAL activity of cultures

The results presented in Fig 3.2.10 a. show that the PAL activity was significantly higher in cultures growing at the lowest nitrate concentration than in medium without nitrate, while both had significantly greater PAL activities than cultures at the remaining concentrations. This pattern of response was noticeable at each subculture although more so at eight weeks (Fig 3.2.10 b).

In Fig 3.2.10 c. it can be seen that there was a consistent and significant drop in PAL activity with increasing subculture until the last at sixteen weeks. This fall in PAL activity was observed in all cultures although there was a slight increase in activity in those growing at the lowest concentration after eight weeks (Fig 3.2.10 d).

3.2.4.5 Summary of the effect of nitrate on the RGR, TCC and PAL activity of suspension cultures

Varying the nitrate concentration caused changes in the appearance, RGR, TCC and PAL activity of the suspension cultures over the sixteen weeks. The notable change in appearance occurred in cultures growing at the lowest concentration or in medium without nitrate, where cells became increasingly brown and aggregated. This change was accompanied by a low or negligible RGR or TCC after four weeks, and a low TCC throughout the experiment; however, these cultures were high in PAL activity at the first and second subcultures of the experiment. Cultures growing in the standard or the two highest nitrate concentrations remained yellow and friable with some green cells although those at the highest were noticeably greener towards the end of the sixteen weeks. These characteristics coincided with an unstable but high RGR and a low PAL activity. Cultures at the two highest nitrate concentrations had a high TCC especially over the latter half of the experiment.

Figure 3.2.9

The effect of nitrate concentration (mg l^{-1}) and subculture on the total chlorophyll content (TCC) (µg g DW^{-1}) of suspension cultures over sixteen weeks. The histograms a,c show the overall effects of treatment and subculture respectively, and the line graphs b,d show the individual effects of subculture at each concentration, and concentration at each subculture time respectively. Error bars represent the LSD values which were determined from the analysis of variance shown in Table 13 of the Appendix.

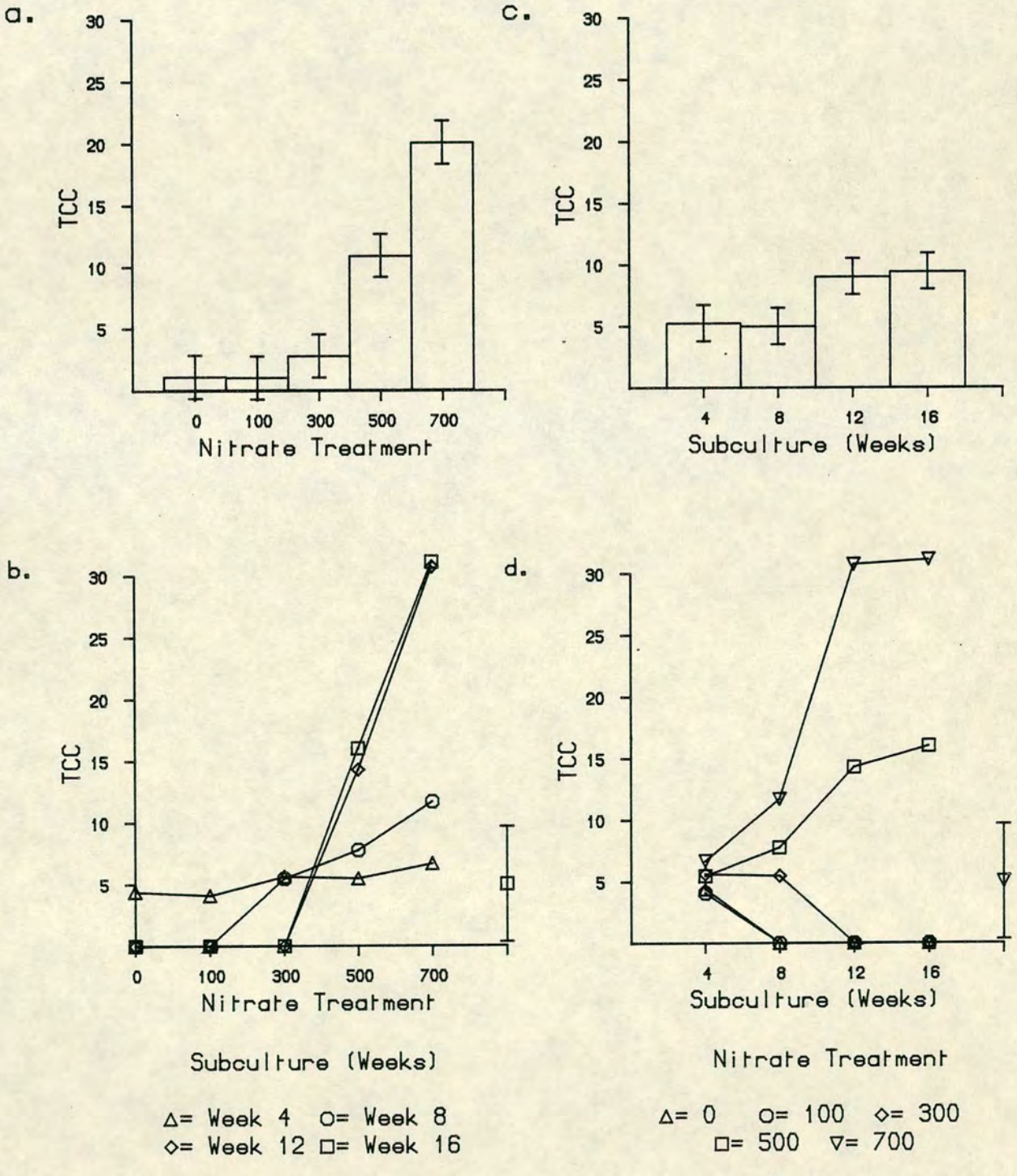
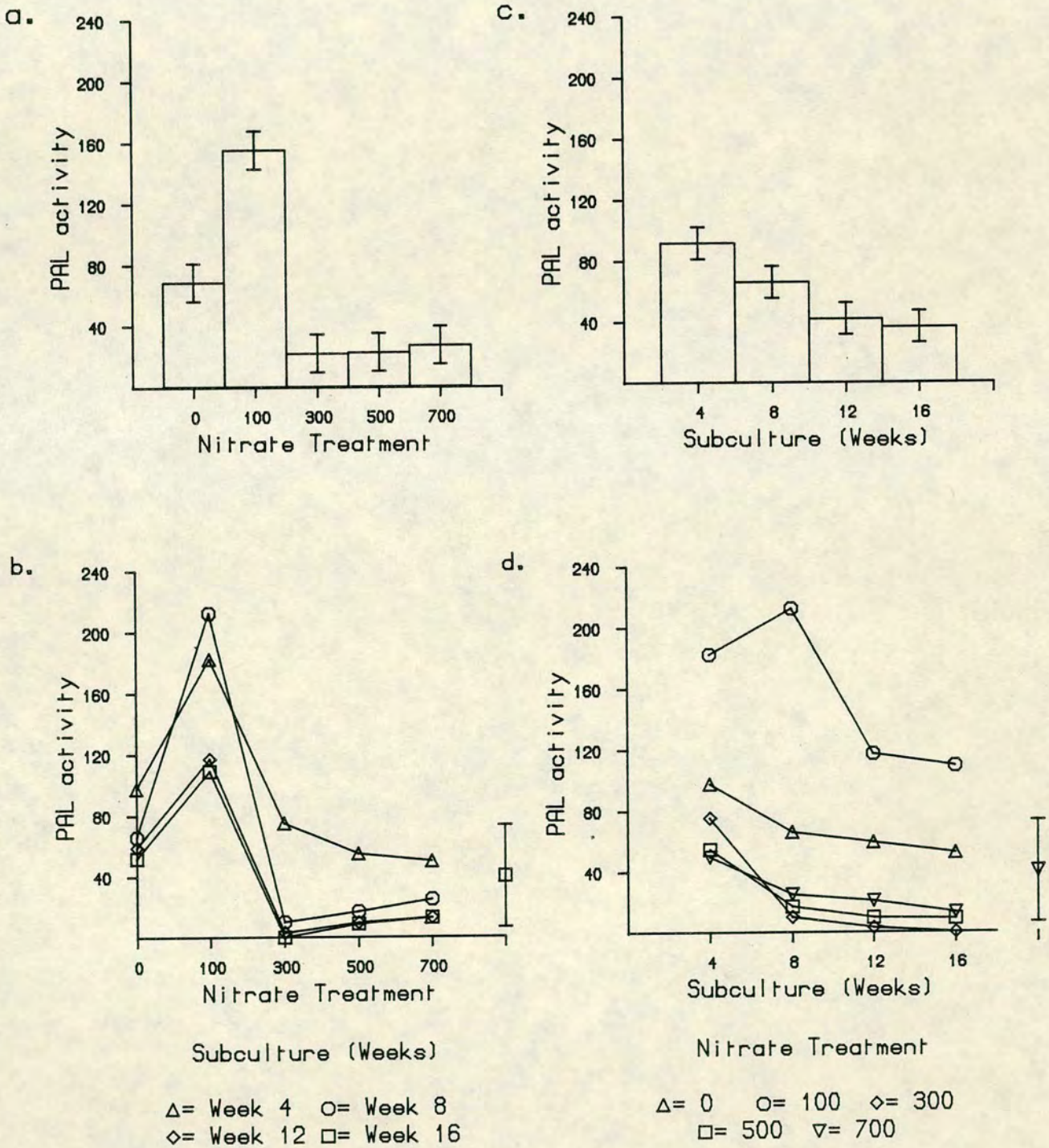


Figure 3.2.10

The effect of nitrate concentration (mg l^{-1}) and subculture on the phenylalanine ammonia lyase activity (PAL) (µkat kg^{-1}) of suspension cultures over sixteen weeks. The histograms a,c show the overall effects of treatment and subculture respectively, and the line graphs b,d show the individual effects of subculture at each concentration, and concentration at each subculture time respectively. Error bars represent the LSD values which were determined from analysis of variance shown in Table 13 of the Appendix.



3.2.5 The effect of phosphate on the morphological and metabolic characteristics of suspension cultures

3.2.5.1 Appearance of cultures

It can be seen from Table 3.2.5 that there were few differences in the appearance of suspension cultures growing in different phosphate concentrations. Cultures growing at the lowest concentration or in medium without phosphate became brown and aggregated after the last subculture (Table 3.2.5). Cultures maintained in the other three concentrations remained mainly yellow and friable with some green cells, although cultures in the standard concentration became mostly green at the end of the sixteen weeks (Table 3.2.5).

3.2.5.2 Relative growth rate (RGR) of cultures

The results presented in Figs 3.2.11 a. show that the RGR of cultures in medium without phosphate was significantly lower than the others. However, this effect was not apparent at the first subculture when the RGR of all cultures was similar (Fig 3.2.11 b).

In Fig 3.2.11 c. it can be seen that the RGR of the cultures was significantly higher at four weeks than after any of the subsequent subcultures. However this effect of subculture was not observed in all cultures as it was found that the RGR of those growing in the two highest phosphate concentrations appeared to increase slightly with subculture although cultures in the other two concentrations, and especially those in the lowest, were not as stable (Figs 3.2.11 d).

3.2.5.3 Total chlorophyll content (TCC) of cultures

The results presented in Fig 3.2.12 a. show that the TCC was significantly higher in cultures maintained at the standard and the two highest phosphate concentrations. However, after the first subculture cells growing in the lowest phosphate concentration or in medium without phosphate had a similar TCC to the others (Figs 3.2.12 b).

Table 3.2.5

The effect of phosphate concentration (mg l^{-1}) on the appearance of suspension cultures over sixteen weeks. Each score is a mean of three sets of observations.

Parent Y(G),F

		Subculture (Weeks)			
	Phosphate Conc.	**Week 4**	**Week 8**	**Week 12**	**Week 16**
	0	Y(G),F(A)	Y(W),A	Y(B),A	B,A
	20	Y(G),F	Y(G),F	Y(B),F	B,A
*	40	Y(G),F(A)	Y(G),F	Y(G),F	G(Y),F
	60	Y(G),F	Y,F	Y(G),F	Y(G),F
	80	Y(G),F(A)	Y(G),F(A)	Y(G),F	Y(G),F

* denotes the concentration used in standard SH medium

Y=Yellow

G=Green

B=Brown

W=White

F=Friable

A=Aggregated

V=Variegated

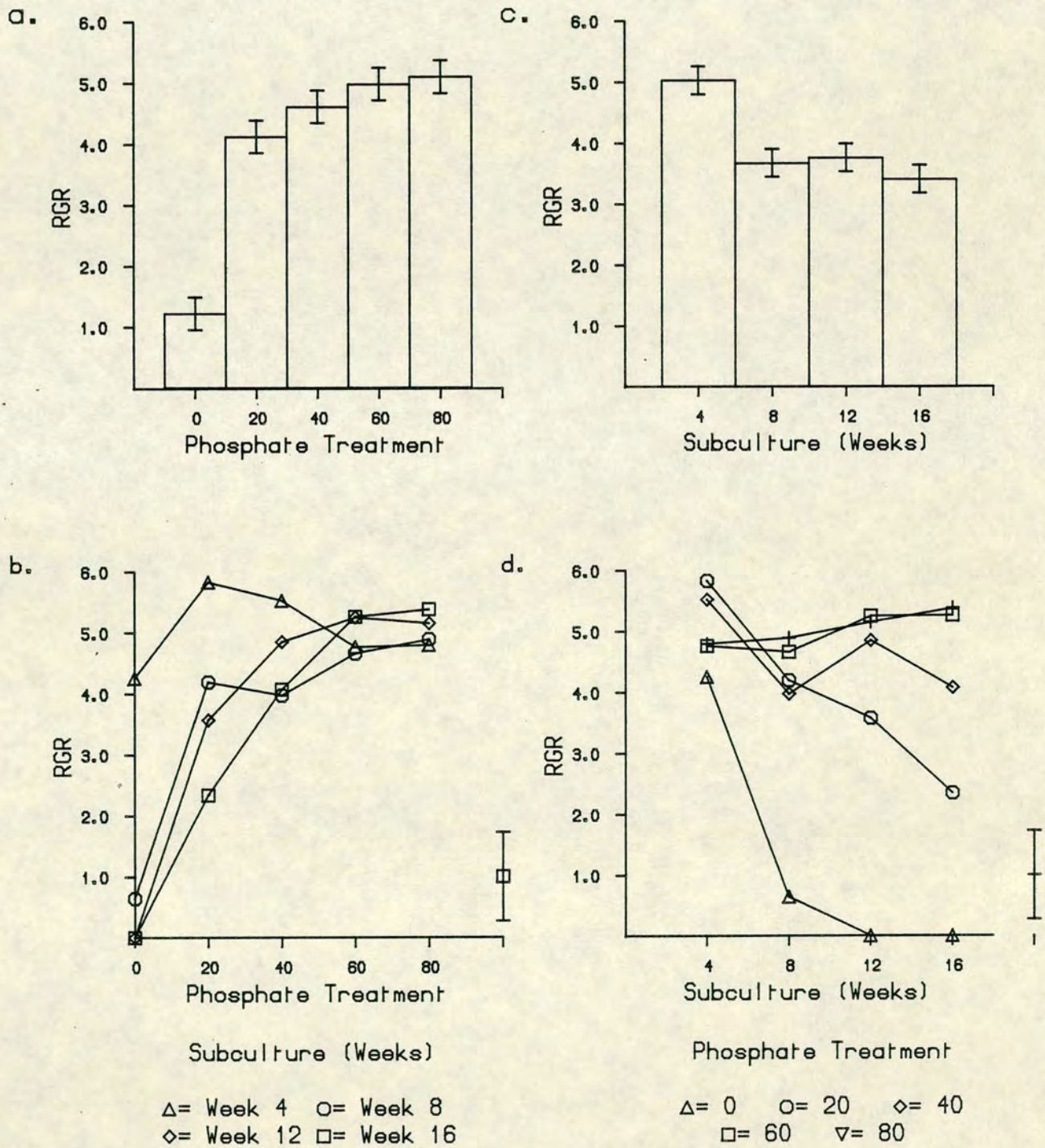
Y(G) means culture mainly yellow with the presence of a small amount of cells of one other colour, in this case green.

Y(V) means culture mainly yellow with the presence of small amounts of other coloured cells (in this case white, green or brown) giving the culture a variegated appearance.

F(A) means culture mostly friable with small amounts of aggregated cells.

Figure 3.2.11

The effect of phosphate concentration (mg l^{-1}) and subculture on the relative growth rate (RGR) ($day^{-1}x10^{-2}$) of suspension cultures over sixteen weeks. The histograms a,c show the overall effects of treatment and subculture respectively, and the line graphs b,d show the individual effects of subculture at each concentration, and concentration at each subculture time respectively. Error bars represent the LSD values which were determined from the analysis of variance shown in Table 14 of the Appendix.



In Fig 3.2.12 c. it can be seen that the effect of increasing subculture had no effect on the TCC in the cultures, however, this pattern of response was not observed in cultures at the lowest concentration and in medium without phosphate as the TCC fell rapidly after the first subculture (Fig 3.2.12 d).

3.2.5.4 PAL activity of cultures

The results presented in Figs 3.2.13 a. and b. show that there was a significantly higher PAL activity in cultures maintained in medium without phosphate, a response which was particularly apparent over the first twelve weeks.

In Fig 3.2.13 c. and d. it can be seen that there was a drop in PAL activity after the last subculture however, the significance of this reduction was accounted for by the greater fall in PAL activity in cultures maintained in medium without phosphate or in medium with 40 mg l^{-1} phosphate.

3.2.5.5 Summary on the effect of different phosphate concentrations on the RGR, TCC and PAL activity of the suspension cultures

Varying the phosphate concentration caused changes in the appearance, RGR, TCC and PAL activity of the suspension cultures over the sixteen weeks. The increased browning and aggregation of cultures maintained at the lowest concentration or in medium without phosphate was accompanied by a consistent and significant drop in RGR and TCC. In contrast, the yellow-green and friable cultures in media containing the standard or the two highest phosphate concentrations had a higher and stable RGR and TCC. The PAL activity was low in all cultures apart from those growing in medium without phosphate where the activity was high for most of the experiment.

Figure 3.2.12

The effect of phosphate concentration (mg l^{-1}) and subculture on the total chlorophyll content (TCC) (µg g DW^{-1}) of suspension cultures over sixteen weeks. The histograms a,c show the overall effects of treatment and subculture respectively, and the line graphs b,d show the individual effects of subculture at each concentration, and concentration at each subculture time respectively. Error bars represent the LSD values which were determined from the analysis of variance shown in Table 14 of the Appendix.

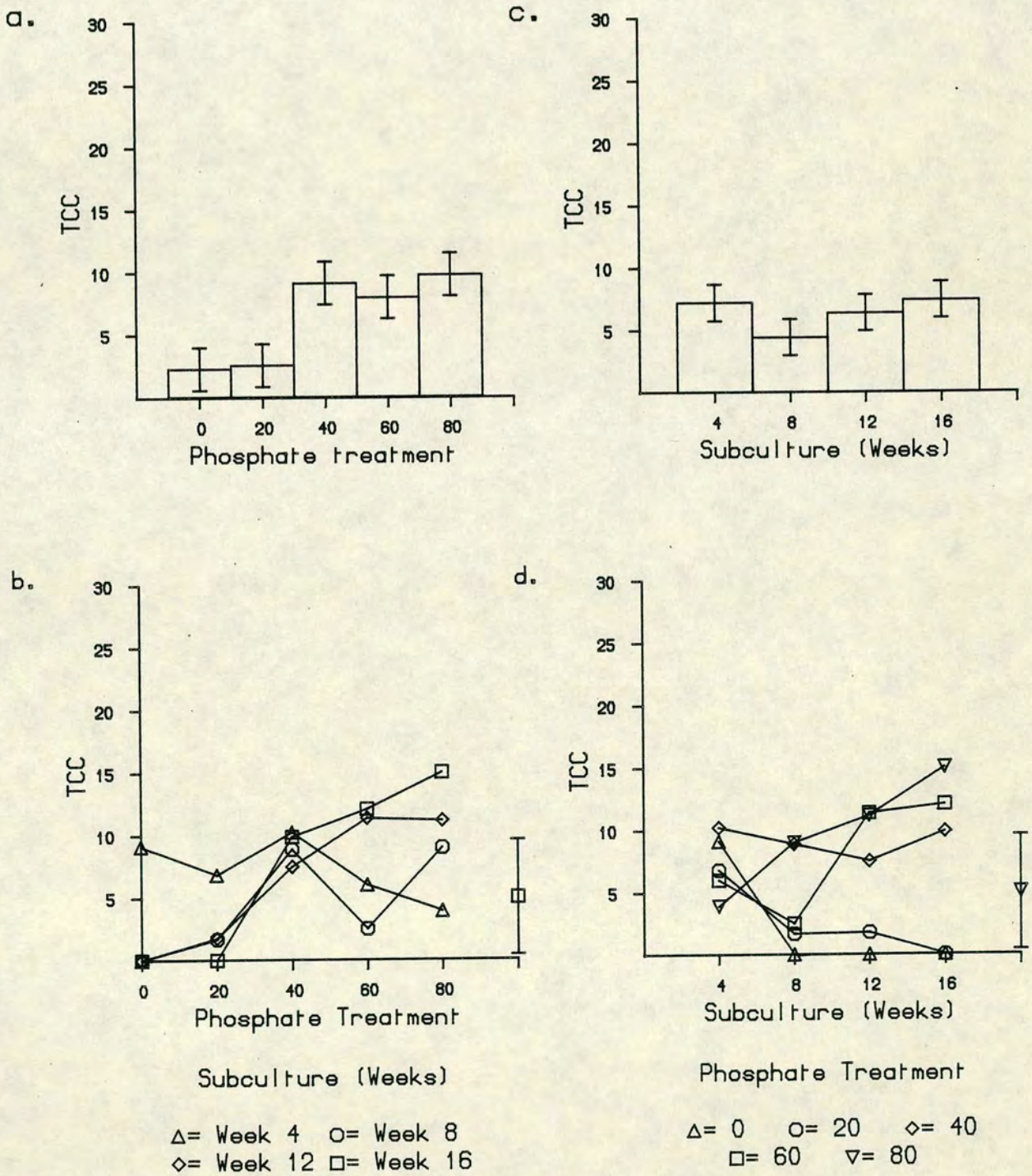
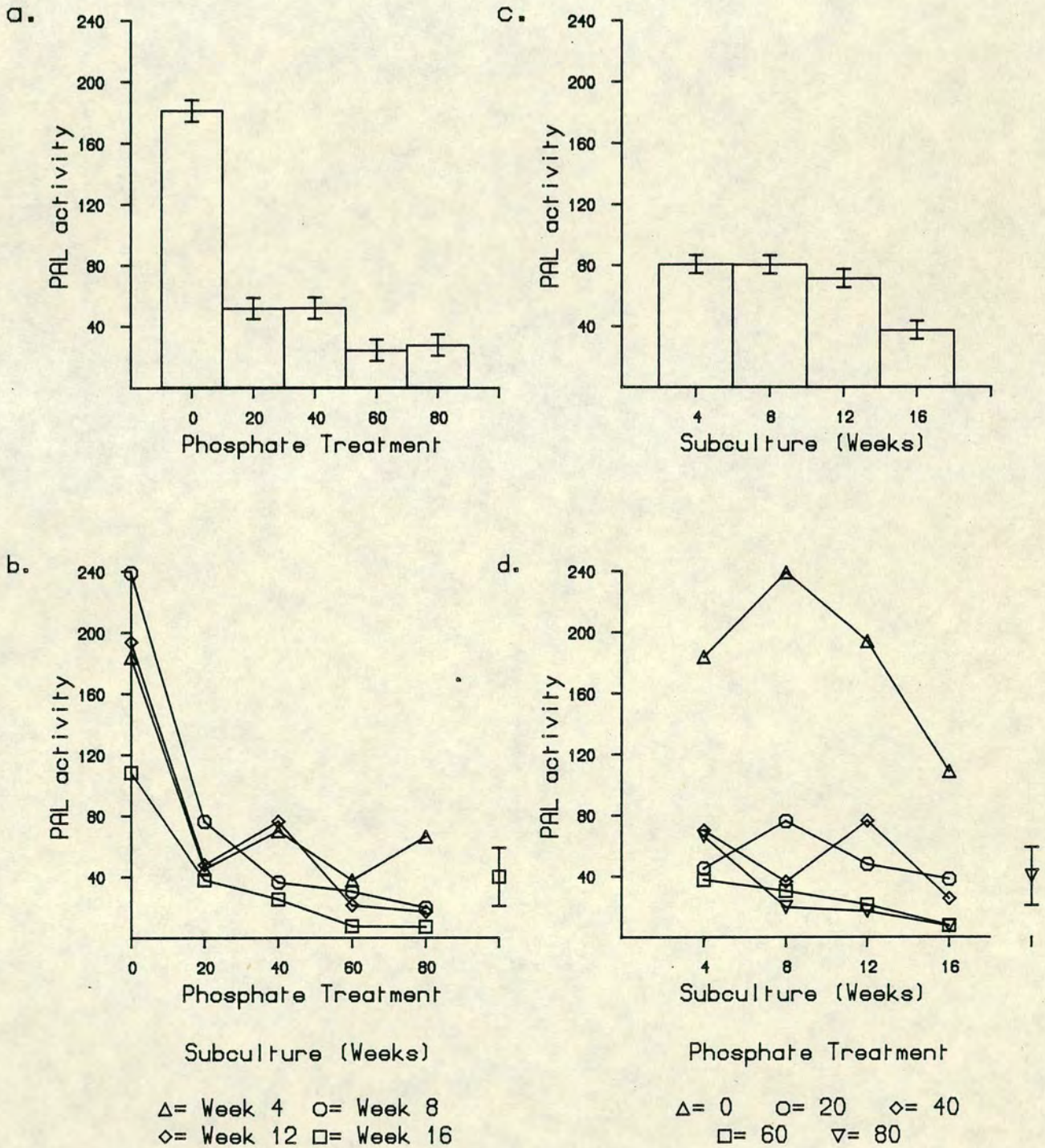


Figure 3.2.13

The effect of phosphate concentration (mg l^{-1}) and subculture on the phenylalanine ammonia lyase activity (PAL) (µkat kg^{-1}) of suspension cultures over sixteen weeks. The histograms a,c show the overall effects of treatment and subculture respectively, and the line graphs b,d show the individual effects of subculture at each concentration, and concentration at each subculture time respectively. Error bars represent the LSD values which were determined from analysis of variance shown in Table 14 of the Appendix.



3.2.6 The effect of pH on the morphological and metabolic characteristics of suspension cultures

3.2.6.1 Appearance of cultures

It can be seen from Table 3.2.6 that there were few changes in the appearance of the suspension cultures maintained at different pHs. All cultures maintained at the standard or the two highest pH levels remained mainly yellow and friable, however, cultures at the two lowest pH levels although remaining yellow-green and friable for twelve weeks became brown and aggregated after the last subculture (Table 3.2.6).

3.2.6.2 Relative growth rate (RGR) of cultures

The results presented in Figs 3.2.14 a. and b. show that there were no significant differences in the RGR among cultures growing at different pHs, and that this pattern was observed after each subculture in the experiment.

In Figs 3.2.14 c. and d. it can be seen that there was a significantly higher RGR at the third and fourth subcultures after twelve and sixteen weeks, a response that was found in cultures at each pH.

3.2.6.3 Total chlorophyll (TCC) content of the cultures

The results presented in Figs 3.2.15 a. and b. show that there were no significant differences in the TCC among cultures growing at different pHs, and that this pattern was observed after each subculture in the experiment.

In Figs 3.2.15 c. and d. it can be seen that there was no effect of subculture on the TCC of the cultures and that this response was found in cultures at each pH.

Table 3.2.6

The effect of pH on the appearance of suspension cultures over sixteen weeks. Each score is a mean of three sets of observations.

Parent Y(G),F

		Subculture (Weeks)			
	pH level	**Week 4**	**Week 8**	**Week 12**	**Week 16**
	4.8	G(Y),(F)A	Y(G),F(A)	Y(G),Fr(A)	B(Y),A
	5.4	Y,F(A)	Y(G),F(A)	Y(G),F(A)	B,F(A)
*	5.8	Y,F(A)	Y(G),F(A)	Y,F(A)	Y,F(A)
	6.2	Y,F	Y,F(A)	Y,F(A)	Y(G),F
	6.8	Y,F(A)	Y,F(A)	Y,F(A)	Y(V),B

* denotes the concentration used in standard SH medium

Y=Yellow

G=Green

B=Brown

W=White

F=Friable

A=Aggregated

V=Variegated

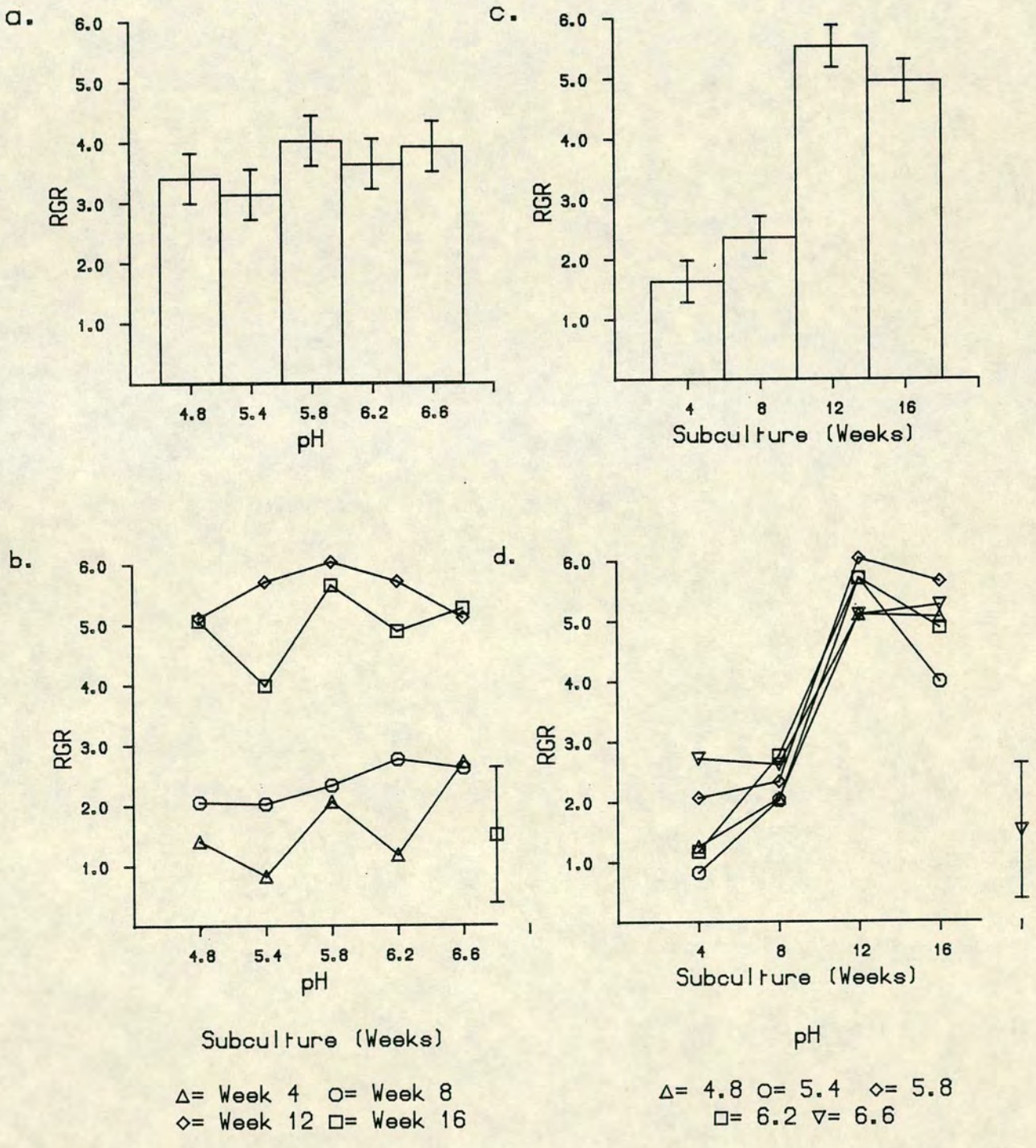
Y(G) means culture mainly yellow with the presence of a small amount of cells of one other colour, in this case green.

Y(V) means culture mainly yellow with the presence of small amounts of other coloured cells (in this case white, green or brown) giving the culture a variegated appearance.

F(A) means culture mostly friable with small amounts of aggregated cells.

Figure 3.2.14

The effect of pH and subculture on the relative growth rate (RGR) (day^{-1} $x10^{-2}$) of suspension cultures over sixteen weeks. The histograms a,c show the overall effects of treatment and subculture respectively, and the line graphs b,d show the individual effects of subculture at each concentration, and concentration at each subculture time respectively. Error bars represent the LSD values which were determined from the analysis of variance shown in Table 15 of the Appendix.



3.2.6.4 PAL activity of the cultures

The results presented in Figs 3.2.16 a. and b. show that there were no significant differences in the PAL activity among cultures growing at different pHs, and that this pattern was observed after each subculture in the experiment.

In Figs 3.2.16 c. and d. it can be seen that there was a significant fall in the PAL activity of the cultures at the last subculture after sixteen weeks, and that this response was apparent in cultures at every pH.

3.2.6.5 Summary on the effect of different pH levels on the appearance, RGR, TCC and PAL activity of the suspension cultures

It would appear that pH did not cause any marked differences in appearance or any significant changes in the RGR, TCC or PAL activity of the suspension cultures. However, the effect of subculture was significant in causing changes in the RGR and PAL activity of these cultures. The RGR increased significantly after twelve weeks, whereas PAL activity dropped significantly after sixteen weeks in culture.

Figure 3.2.15

The effect of pH and subculture on the total chlorophyll content (TCC) (µg g DW^{-1}) of suspension cultures over sixteen weeks. The histograms a,c show the overall effects of treatment and subculture respectively, and the line graphs b,d show the individual effects of subculture at each concentration, and concentration at each subculture time respectively. Error bars represent the LSD values which were determined from the analysis of variance shown in Table 15 of the Appendix.

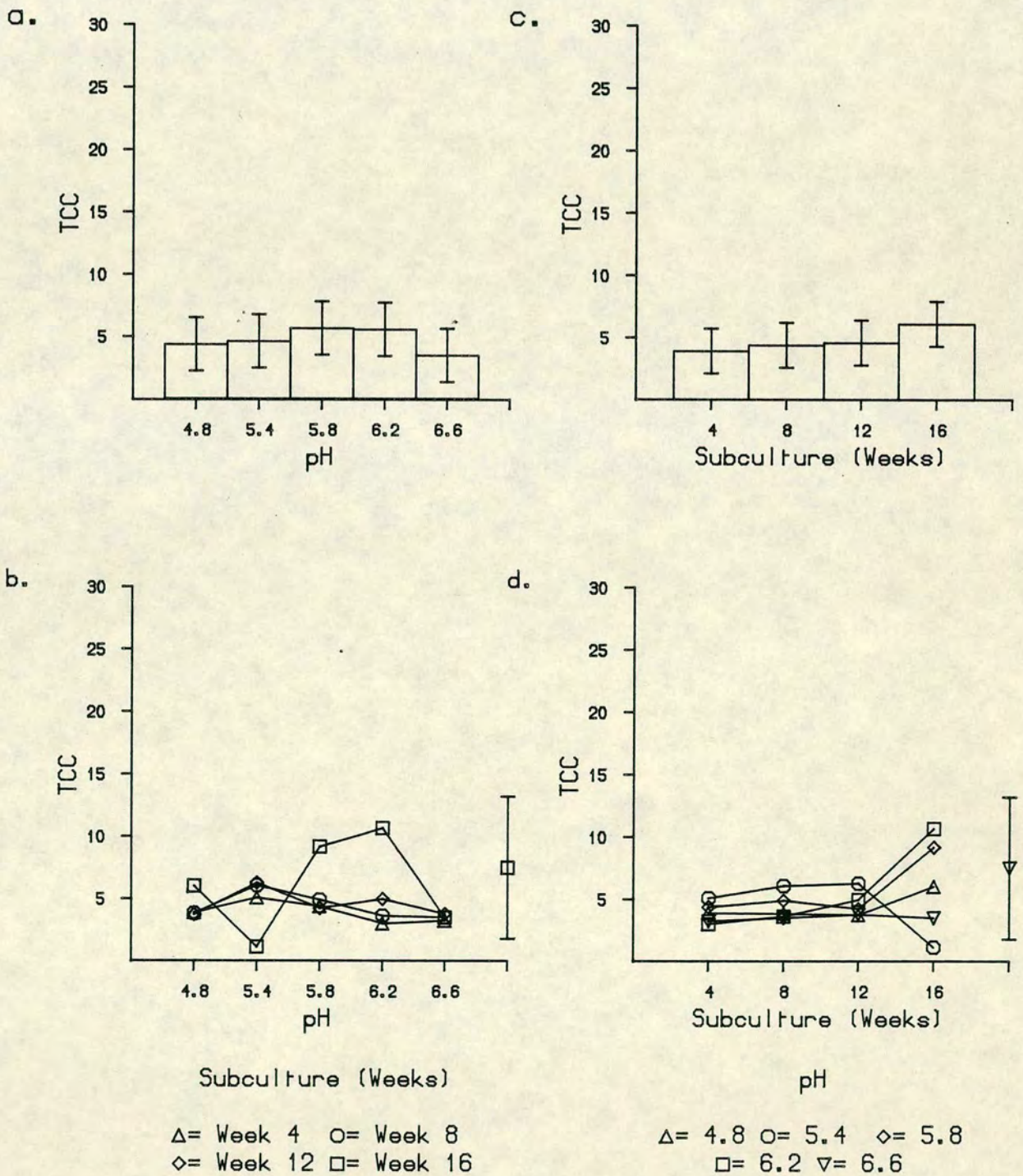
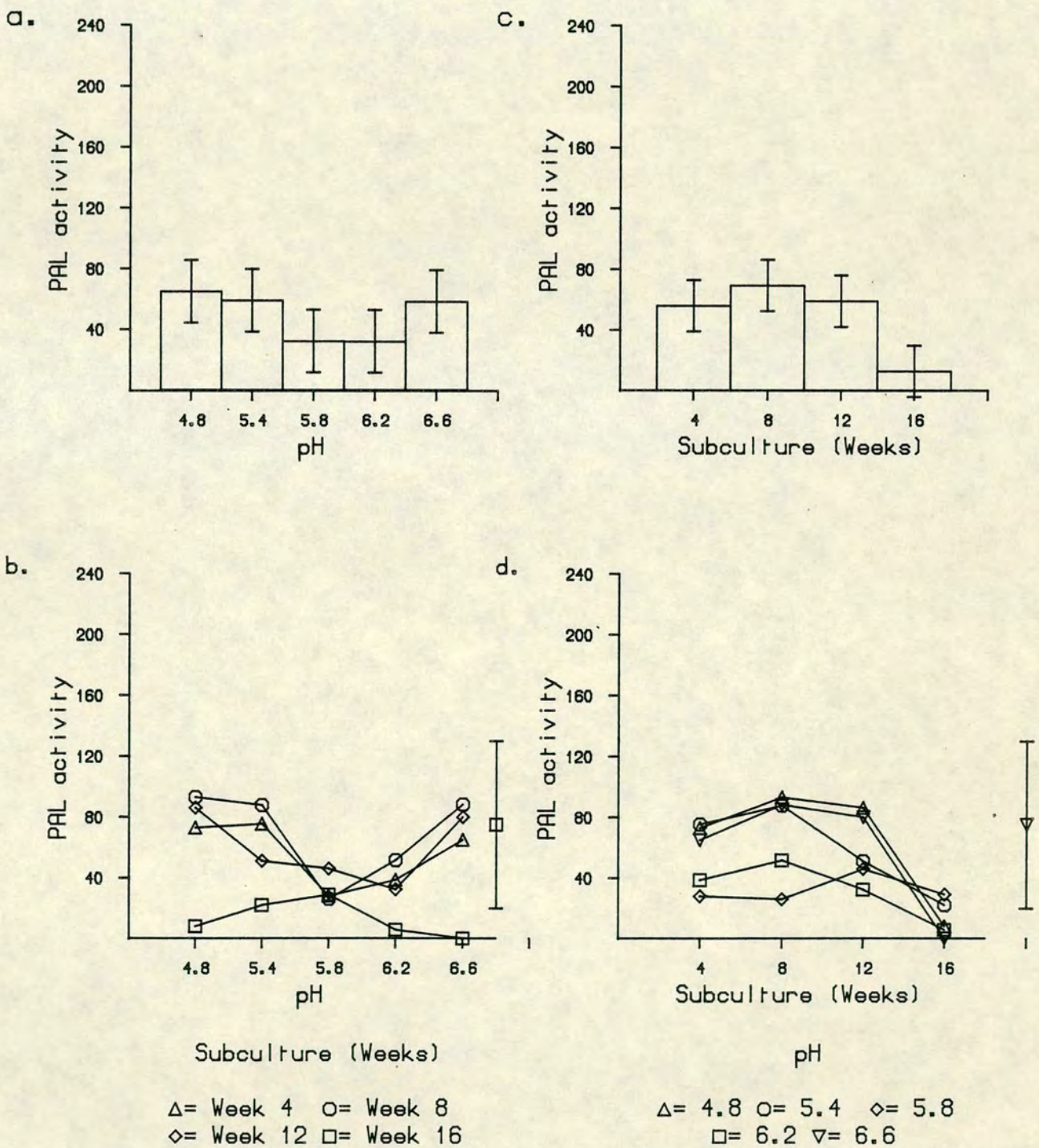


Figure 3.2.16

The effect of pH and subculture on the phenylalanine ammonia lyase activity (PAL) (µkat kg^{-1}) of suspension cultures over sixteen weeks. The histograms a,c show the overall effects of treatment and subculture respectively, and the line graphs b,d show the individual effects of subculture at each concentration, and concentration at each subculture time respectively. Error bars represent the LSD values which were determined from analysis of variance shown in Table 15 of the Appendix.



3.2.7 Conclusions on the effects of medium constituents on the morphology and metabolism of suspension cultures

In these experiments it has been shown that changes in essential medium constituents cause noticeable and significant changes to arise in morphology and metabolism of suspension cultures over sixteen weeks. Cultures growing in standard conditions usually maintained a yellow-green and friable appearance, high RGR, low to high TCC and a low PAL activity. However, cultures maintained in the other conditions responded to their environment in different ways.

Cultures growing in medium containing low concentrations (less than the standard concentration) of sucrose, nitrate and phosphate became brown and aggregated which coincided with a low and decreasing RGR and TCC, a change that was both immediate and irreversible, in cultures without the specific component. However, the appearance, RGR and TCC of the cultures growing in low concentrations of 2,4-D or at low pH did not appear to be affected, infact cultures were found to grow appreciably and accumulate chlorophyll in media without 2,4-D suggesting that these cultures were capable of auxin independent growth. The PAL activity was high in those cultures with low concentrations of nitrate and phosphate which goes some way to suggest that the enzyme was stimulated by the morphological state of the cultures rather than the treatment concentrations themselves. In this respect, slow growing and aggregated cultures did correspond with higher PAL activities.

In general, cultures growing at the two highest concentrations of all the medium components had either a similar to or greater than response to those growing in the the standard medium. However, in the 2,4-D and sucrose treatments the values found at the first and sometimes the second subculture were lower than those from the standard medium due to a possible carry over effect from the previous standard SH medium. Of all the treatments used pH had the least effect on the morphology and metabolism of the cultures. Differences in PAL activity were on the whole inconsistent and the actual values varied considerably among the treatments irrespective of concentration. PAL activity, although apparently associated in some cases with the state of the cultures, in other cases was not, suggesting perhaps inherent differences among the culture lines.

PART THREE

THE EFFECT OF TISSUE ORIGIN ON THE GROWTH AND MORPHOLOGY OF CALLUS CULTURES

3.3 The effect of tissue origin on the growth and morphology of callus cultures

The physiological and developmental state of explanted cells is known to affect the morphological and biochemical responses of resulting cultures (Zenk *et al.* 1977, Lindsey and Yeoman 1985, Mikami and Kinoshita 1988). Therefore the choice of explant is important as it can influence the initiation and growth of cultures, and must be taken into consideration when the establishment of a tissue culture is contemplated.

This series of experiments was carried out to examine the influence of explant origin on the establishment and morphology of resultant callus cultures. It involves comparisons among callus cultures derived from;

1. different parts of the same plant
2. three plants from the same age group
3. four plants that differed in age.
4. five plants that differed in genotype.

3.3.1 Response of cultures from different parts of the same plant

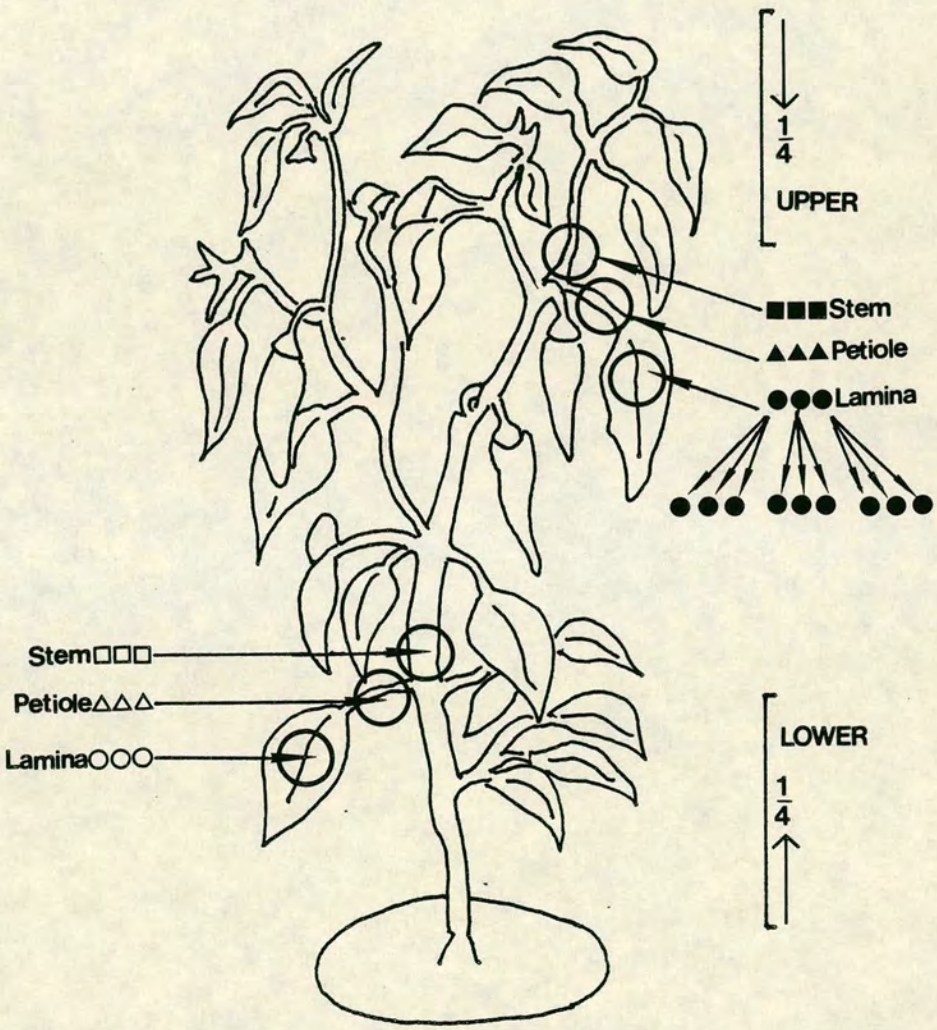
In this experiment the relative growth rates (RGR) of callus cultures derived from different parts of the same plant were determined and compared over sixteen weeks.

3.3.1.1 Experimental procedure (see Fig 3.3.1)

A single, healthy individual of *Capsicum frutescens* cv. Cayenne was selected from a group of four-month old plants. It was measured from soil level to apex, and the regions upper and lower were designated respectively as one quarter of this distance from the apex down, or one quarter from the base upwards. Three separate parts of stem and leaf (petiole and lamina) were excised from both these regions.

Figure 3.3.1

Diagram showing the procedure for the excision of stem, petiole amd lamina tissue from the upper and lower regions of a 4-month old plant of *Capsicum frutescens* cv. Cayenne.



Each part was sealed with wax and sterilized using the procedures described in Section 2.2. After sterilization the petiole was separated from the leaf lamina. Each of the three stem and petiole parts were then cut into three equal sized pieces (1cm. long) while from each of the three lamina parts three equal sized discs (1cm. in diam.) were punched out. These nine stem, petiole and lamina pieces were then transferred to the surface of standard SH medium in 9 cm. Petri dishes. The explanted material was then left to callus for four weeks under normal growth conditions after which each of the nine replicate calluses was excised from the explant and transferred to fresh SH medium. These calluses were maintained for sixteen weeks with a subculture every four weeks at which time the RGR was determined. Apart from the first analysis in this experiment, in which the nine values were grouped as three sets of three replicates, all nine cultures were treated as replicates. All the RGR results were subjected to an analysis of variance to determine any significant differences after which the following comparisons were made;

1. Within each group of stem, petiole and lamina calluses from the upper region using the three sets of three replicates.
2. Among stem, petiole and lamina calluses within the upper or lower regions using all nine cultures as replicates.
3. Between stem, petiole and lamina calluses from the upper and lower regions using all nine cultures as replicates.

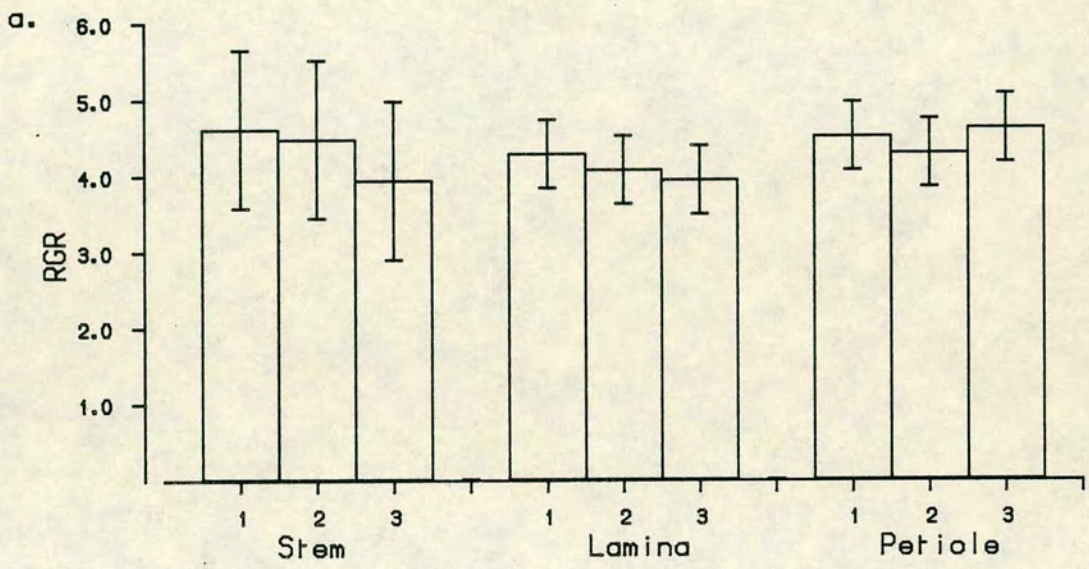
3.3.1.2 Relative growth rate (RGR) of callus cultures derived from different parts of the same region and from different regions of the same plant

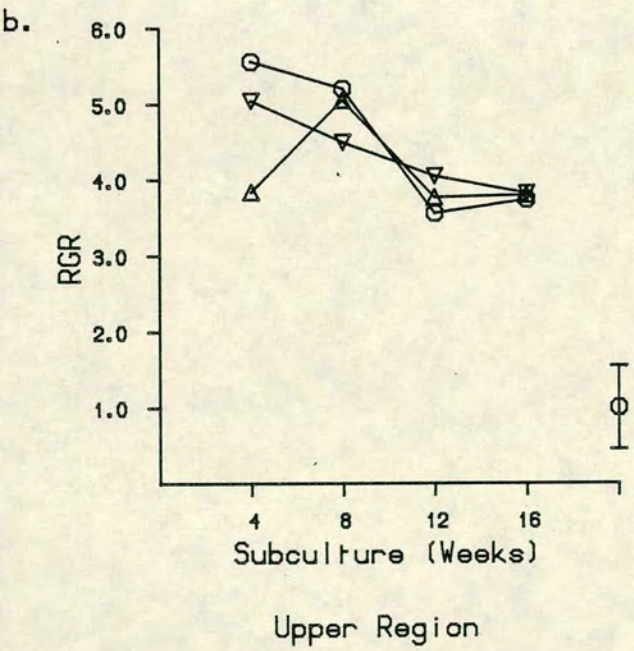
It can be seen in Fig 3.3.2 a. that there were no significant differences in RGR among the three groups of three callus cultures of either stem, petiole or lamina from the upper region of the plant over sixteen weeks.

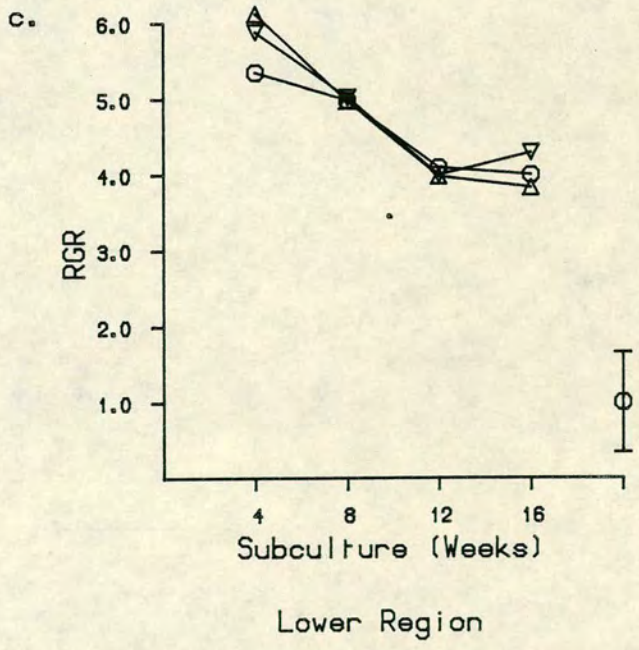
The results presented in Fig 3.3.2 b. show that there were no significant differences in RGR among cultures derived from the stem, petiole or lamina from the upper region of the plant over sixteen weeks, apart from a significantly lower RGR in lamina cultures at four weeks. Similarly, there were no significant differences among the RGR of cultures from stem, petiole or lamina from the lower region of the plant over sixteen weeks (Fig 3.3.2 c).

Figure 3.3.2

Comparison of relative growth rate (RGR) ($day^{-1}x10^{-2}$) of callus cultures of stem, lamina and petiole derived from (a). among replicate pieces from the upper region and among parts from the (b). upper and (c). lower region of a single plant over sixteen weeks. The error bars represent the LSD values which were determined from the analysis of variance shown in Tables 16 and 17 of the Appendix.







These results also show that apart from the significant increase in RGR of lamina cultures from the upper region at eight weeks the RGR fell significantly in all cultures over sixteen weeks (Figs 3.3.2 b,c).

A comparison in RGR of callus cultures from stem, petiole and lamina between the upper and lower regions of the plant shows that there were no significant differences in the stem and petiole cultures between these two regions (Figs 3.3.2 b,c). However, cultures from the lower lamina had a significantly greater RGR than those from the upper region, a response that could be accounted for by the values found at the first subculture after four weeks (Figs 3.3.2 b,c).

3.3.2 Response of cultures from three plants of the same age group

In this experiment the RGR of callus cultures from the stem of three individual plants within the same group were determined and compared.

3.3.2.1 Experimental procedure

Three plants (cv.Cayenne) A, B and C were selected from a group of four-month old plants (Table 3.3.1). Nine calluses derived originally from three stem parts from the upper region of each plant were established using the procedure described in the last experiment. These cultures were maintained for sixteen weeks with a subculture and a determination of RGR every four weeks. The RGR values were used in an analysis of variance to determine significant differences.

3.3.2.2 Relative growth rate of callus cultures

It can be seen from Figs 3.3.3 a,b that there were no significant differences in the RGR of callus cultures derived from the three individual plants at any of the subculture times. However, the results show that there was a significant drop in RGR after the first subculture at four weeks which was particularly marked in the stem cultures of plant B (Figs 3.3.3 c,d).

Table 3.3.1

A comparison of three individual plants within a group of four month old plants of *Capsicum frutescens* cv. Cayenne.

Plant	Plant Appearance
Plant A	Green, branching, and leafy shrub with long red conical fruit. 90 cm. high
Plant B	Green, branching and leafy shrub with short to long conical fruit. 110 cm. high.
Plant C	Green, branching and leafy shrub with long red conical fruit. 90 cm. high

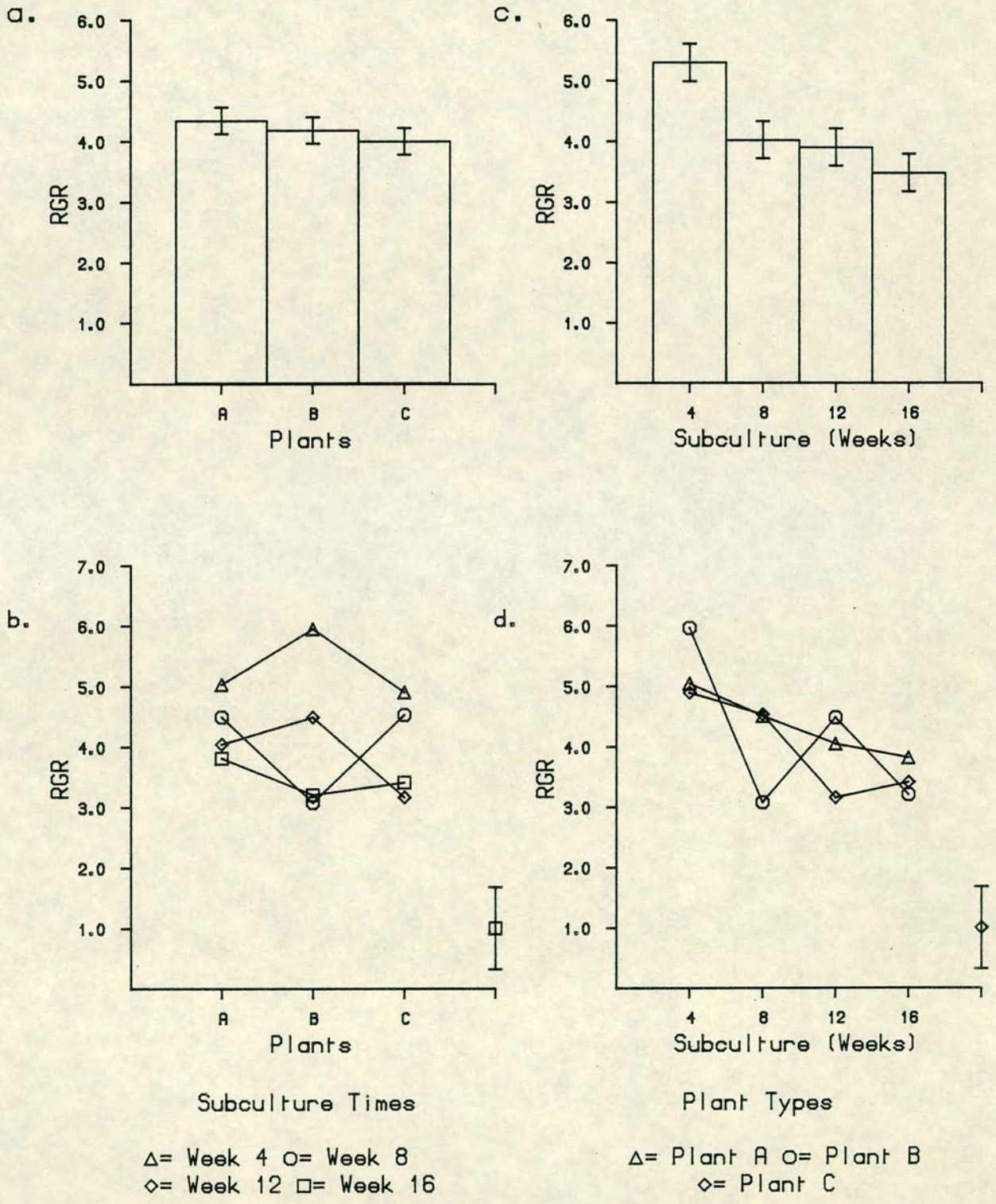
Table 3.3.2

Comparison of individual plants of *Capsicum frutescens* cv. Cayenne from groups of different ages.

Age of Plant	Plant Appearance
2 Months	Green and leafy, no fruit. 25 cm. high
3 Months	Green, branching and leafy, no fruit 60 cm. high.
4 Months	Green, branching and leafy with long red conical fruit. 90 cm. high
5 months	Green, branching, leafy and woody with long red conical fruit. 90 cm. high.

Figure 3.3.3

The effect of plants from the same group and of subculture on the relative growth rate (RGR) ($day^{-1}x10^{-2}$) of stem derived callus cultures over sixteen weeks. The error bars represent the LSD values which were determined from the analysis of variance shown in Table 18 of the Appendix.



3.3.3 Response of cultures from plants of different ages

In this experiment the RGR of callus cultures from the stem of four individual plants of different age was determined and compared over sixteen weeks.

3.3.3.1 Experimental procedure

One plant was selected from each group of two, three, four and five month old plants (cv.Cayenne, age determined from germination) and grown under the conditions described in Section 2.2. Only healthy plants were selected with characteristics representative of the group (Table 3.3.2). The isolation and establishment of the nine callus cultures from three equally sized stem parts from the upper region of the four plants was carried out as described in the first of these experiments.

3.3.3.2 Relative growth rate of callus cultures

It can be seen from Fig 3.3.4 a. that there were no significant differences in the RGR of callus cultures derived from plants of different ages. However, it was noticeable that the cultures from the four-month old plant had a significantly greater RGR at the third subculture after eight weeks (Fig 3.3.4 b). The results presented in Fig 3.3.4 c. show that there was a significant drop in the RGR at the third subculture after twelve weeks. This response to subculture was evident in all cultures although in the four-month old culture this reduction in RGR was even more pronounced because of the high RGR observed at eight weeks (Fig 3.3.4 d).

3.3.4 Response of cultures from plants with different genotypes

In this experiment the appearance and the RGR of callus cultures from the stem of five individual plants with different genotypes was determined and compared over sixteen weeks.

3.3.4.1 Experimental procedure

A plant was selected from each of five groups of four-month old *Capsicum* 'species' which had been grown from seed under the conditions described in Section 2.1.

Figure 3.3.4

The effect of plants of different ages and of subculture on the relative growth rate (RGR) ($day^{-1}x10^{-2}$) of stem derived callus cultures over sixteen weeks. The error bars represent the LSD values which were determined by the analysis of variance shown in Table 18 of the Appendix.

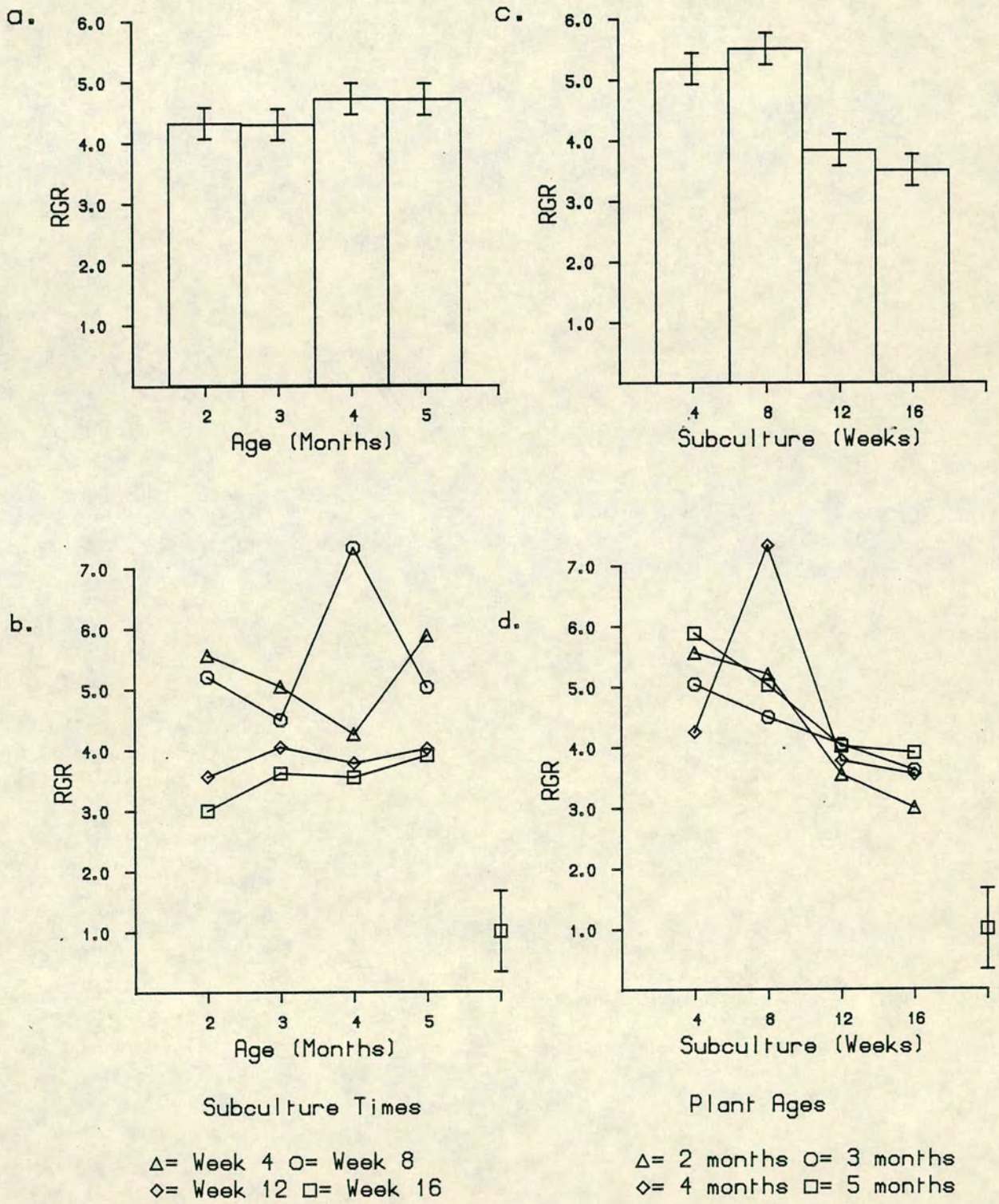


Table 3.3.3

Comparison of individual plants from different 'species' of *Capsicum*. Photographic descriptions are shown in Fig 3.3.5.

Capsicum **Species**	**Plant Appearance**
Capsicum frutescens cv. Cayenne (CF)	Green, woody, prostrate and leafy shrub with long red conical fruit. 90 cm. high
Capsicum baccatum (CB)	Light green, woody, hairy and erect shrub with small red oblong fruit. 240–270 cm. high
Capsicum chinense (CC)	Green, woody and prostrate shrub. Small round red fruit. 45 cm. high
Capsicum frutescens wild type (CFW)	Green, woody and erect shrub. Small red conical fruit. 45 cm. high
Capsicum annuum (CA)	Light green, woody and erect shrub. Small red conical fruit. 150–180 cm. high

Figure 3.3.5

Appearance of individual four-month old plants selected from five different groups of *Capsicum* 'species'.

1. *Capsicum frutescens* cv. Cayenne (CF)
2. *Capsicum annuum* (CA)
3. *Capsicum baccatum* (CB)
4. *Capsicum chinense* (CC)
5. *Capsicum frutescens* (CFW)

1

2

3

4

5

Each 'species'; *Capsicum frutescens* cv. Cayenne (CF), *Capsicum baccatum* (CB), *Capsicum chinense* (CC), *Capsicum annuum* (CA) and *Capsicum frutescens* wild type (CFW), differed considerably in height, colour, habit and fruit size (Table 3.3.3, Fig 3.3.5).

Each selected plant appeared healthy and typical of the sample as a whole. The isolation and establishment of callus from three equally sized stem parts from the upper region of each plant was carried as described in the first of these experiment. Unlike the previous experiments, a record of appearance was also made at every subculture.

3.3.4.2 Appearance of callus cultures

The callus cultures of CF became yellow and friable after eight weeks and remained this way to the end of the experiment (Table 3.3.4, Fig 3.3.6 a). The other four species changed progressively in both colour and texture (Table 3.3.4, Fig 3.3.6 b–e). Brown cells were observed in cultures of CA and CC, while yellow and green cells were apparent in CB and CFW after sixteen weeks (Table 3.3.4, Fig 3.3.6). Also by the end of the experiment aggregates were found in CC, CFW and CA while cultures of CB and CC were still friable (Table 3.3.4, Fig 3.3.6).

3.3.4.3 Relative growth rate of callus cultures

The results presented in Fig 3.3.7 a. show that the RGR of callus cultures from the two genotypes CC and CB were significantly greater than the RGR of cultures from CA and CFW, while CB cultures had a significantly greater value than all but one of the genotypes and CFW had a significantly lower RGR than all genotypes. These differences in RGR were noticeable after each subculture time, although the RGR of CB cultures was markedly higher at four weeks than the other cultures at this time (Fig 3.3.7 b).

These results also show that the RGR of cultures fell significantly at the third subculture after twelve weeks, a result that was particularly evident in CFW and CA (Fig 3.3.7 d). However, examination of the effect of subculture within the individual culture types shows that the RGR of CC cultures remained stable throughout the sixteen weeks (Fig 3.3.7 d).

Table 3.3.4

Appearance of callus cultures derived from stem parts of five 'species' of *Capsicum* over sixteen weeks. Each score is an average of nine separate observations.

	Appearance			
Genotype	**Week 4**	**Week 8**	**Week 12**	**Week 16**
Capsicum frutescens (CF)	Y(W),F	Y,F	Y,F	Y,F
Capsicum baccatum (CB)	Y,F	Y(O),F	Y(O),F	Y(O),F
Capsicum chinense (CC)	Y(W),F	Y(W),F	Y(V),F(A)	B(V),F(A)
Capsicum frutescens wild type (CFW)	Y(W),F	Y,A	Y(V),A	G(V),A
Capsicum annuum (CA)	Y,F	Y(B),F(A)	B(G),A(F)	B(G),A(F)

Y=Yellow

G=Green

B=Brown

W=White

F=Friable

A=Aggregated

V=Variegated

O=Orange

Y(G) means culture mainly yellow with the presence of a small amount of cells of one other colour, in this case green.

Y(V) means culture mainly yellow with the presence of small amounts of other coloured cells (in this case white, green or brown) giving the culture a variegated appearance.

F(A) means culture mostly friable with small amounts of aggregated cells.

Figure 3.3.6

Appearance of callus cultures derived from stem parts of five 'species' of *Capsicum* at the end of a sixteen week period.

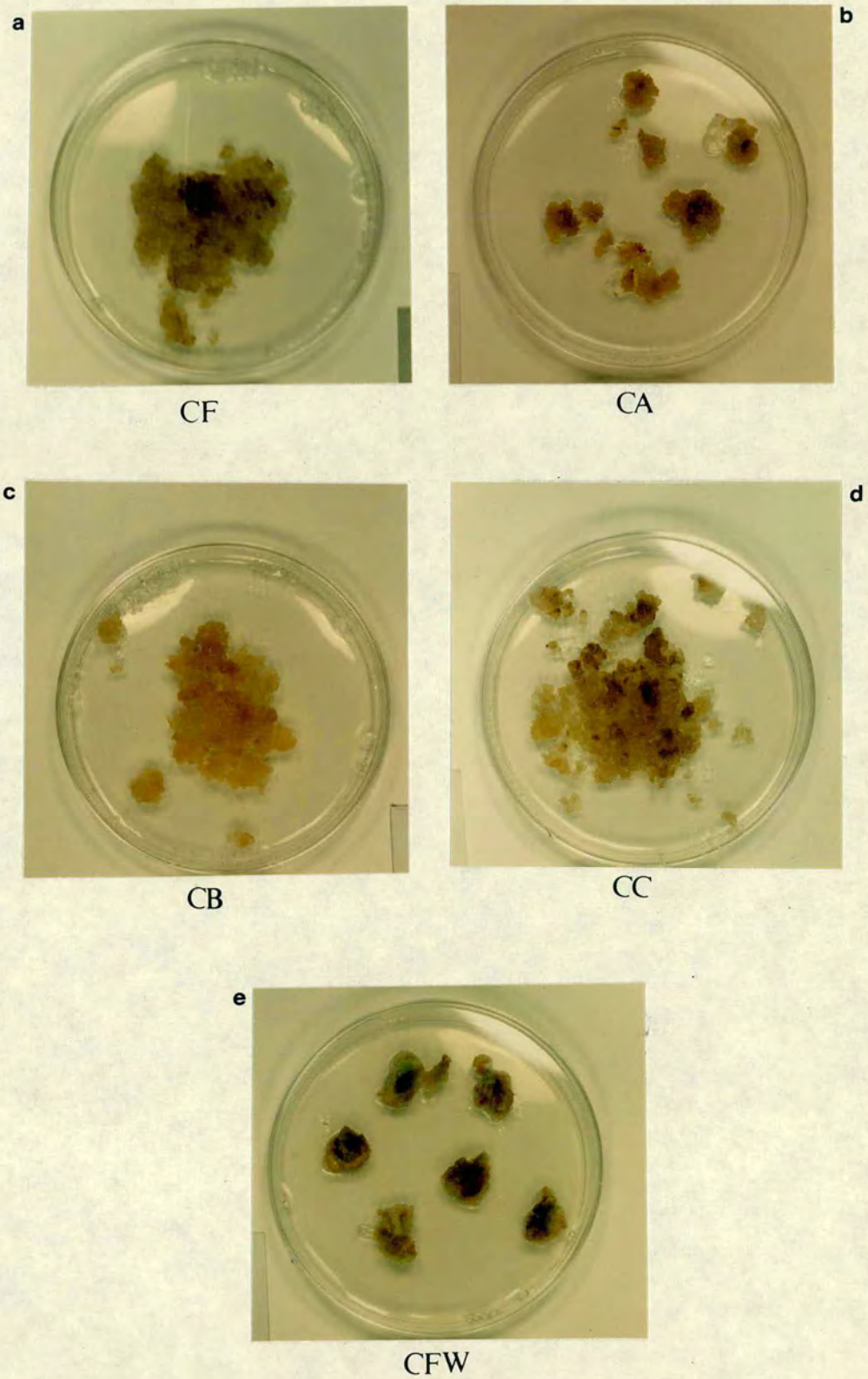
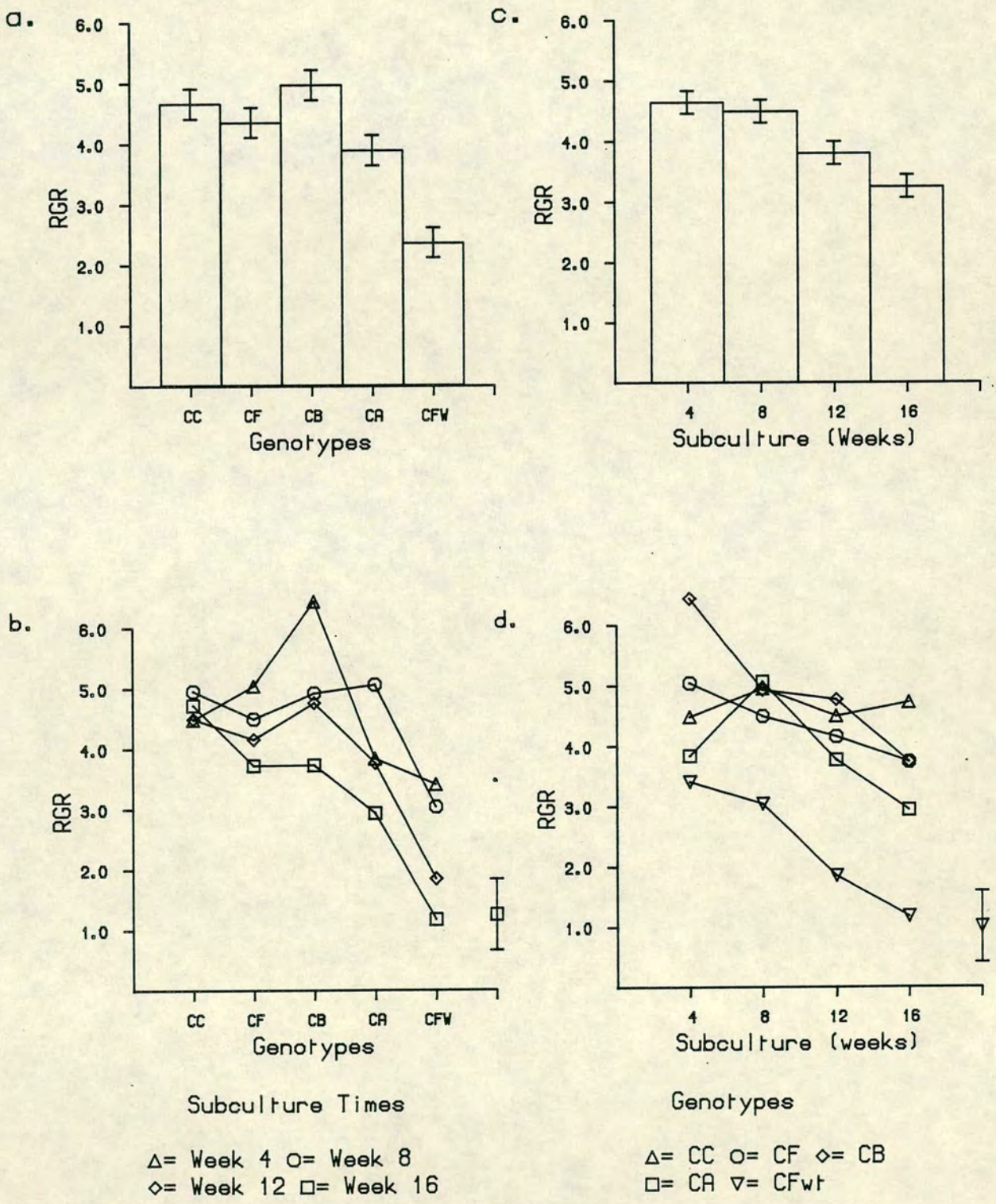


Figure 3.3.7

The effect of plants of different species on the relative growth rate (RGR) ($day^{-1}x10^{-2}$) of stem derived callus cultures over sixteen weeks. The error bars represent the LSD values which were determined by the analysis of variance shown in Table 18 of the Appendix.



3.3.5 Conclusions on the effect of tissue origin on the growth and morphology of callus cultures

There were no significant differences in the RGR among callus cultures derived from different pieces of the same or different parts within the upper and lower regions of the plant over the sixteen weeks. Although there were no significant differences in the RGR of callus cultures of stem and petiole between the upper and lower regions of the plant, there were significantly greater RGR values in the cultures derived from the lower lamina especially after the first four weeks in culture. The RGR of all callus material in the single plant experiment dropped significantly over the sixteen weeks.

There were no significant differences in the RGR of stem derived callus cultures of three plants of the same plant group, however, the RGR of all cultures dropped significantly with subculture particularly in the cultures of plant B. Similarily, the use of plants of different ages as a source of explant material did not cause any significant differences in the RGR of callus cultures, although there was a significant drop in the RGR of the cultures after eight weeks.

The use of different *Capsicum* 'species' with contrasting growth habits, as the source material for the isolation and maintainance of callus cultures, resulted in marked changes in the appearance and significant changes in the RGR of the callus cultures over the sixteen weeks. The callus cultures of CFW and CA became brown or green, and aggregated which was accompanied by a significant drop in RGR especially after the second subculture and significantly lower RGR values over the sixteen weeks. In contrast, the callus cultures of CF and CB remained mainly yellow and friable with a high RGR which, although remaining high, decreased with increasing subculture. However, the callus cultures of CC although turning brown and partially aggregated still retained a consistently high RGR.

PART FOUR

REGENERATION OF CALLUS MATERIAL

3.4 Regeneration of callus material

Another example of variation in culture is the loss of the ability of callus to regenerate. An understanding of this is complicated by the fact that a proper balance of plant growth regulators is needed to induce and sustain regeneration. This series of experiments was carried out to find the best combination of growth regulators for regeneration.

3.4.1 Regeneration of clone AE2 callus

In this first experiment the effect of selected growth regulators on callus of clone AE2 was studied. Two auxins and two cytokinins were used in an attempt to induce regeneration from callus over a 35 day period.

3.4.1.1 Experimental procedure

Callus pieces (*ca.* $1cm^3$ size) were picked from a randomised population and placed onto 10ml. of various SH media, contained in 15ml. Universal tubes, each differing in the type and concentration of growth regulators as shown in Table 3.4.1. The callus was maintained for 35 days and the growth, colour and extent of regeneration (shooting and/or rooting) was scored after 14 days, and subsequently after every 7 days, using an arbitrary scale from 1 to 5 (Table 3.4.2).

3.4.1.2 Effect of growth regulators on the callus of clone AE2

It can be seen from the scores at 35 days that although regeneration did not take place there were differences in the growth and colour of the callus which had emerged over the course of the experiment (Table 3.4.3).

The two auxins induced contrasting responses, NAA usually supported yellow, friable and rapidly proliferating callus while the callus on media containing IAA grew slowly and turned green, both responses being particularly obvious when the auxin was used alone or with 1.0mg l^{-1} 6-BAP (Table 3.4.3). Callus supplemented with either of the two cytokinins became slow growing and those with 6-BAP were noticeably greener (Table 3.4.3). It can be seen from Table 3.4.3 that early signs of root regeneration were observed on medium containing either 1mg l^{-1} 6-BAP or 1mg l^{-1} Kinetin.

Table 3.4.1

Types and concentration (mg l^{-1}) of growth regulators used in experiments on regeneration of callus from clone AE2, stem, lamina and hypocotyl.

Material	Growth Regulators		Concentration
AE2	Auxin	IAA	1.0
		NAA	1.0
	Cytokinin	6-BAP	1.0,2.0
		Kinetin	1.0,2.0
Stem Lamina	Auxin	IAA	1.0,2.0,5.0
	Cytokinin	6-BAP	1.0,2.0
		Kinetin	1.0,2.0
Hypocotyl	Auxin	IAA	1.0, ,5.0,10·0
	Cytokinin	6-BAP	1.0,2.0,5.0,8.0,10.0,12.0
		Zeatin	1.0,2.0,5.0,8.0,10.0,12.0

Table 3.4.2

Arbitrary scale used to determine the stages of regeneration in callus material exposed to different growth regulators.

Character	Scale				
	1	2	3	4	5
Growth	No Growth	Slow	Moderate	Fast	V.Fast
Colour	Brown	White	Yellow	Green	V.Green
Shoots	No Shoots	Pre-Shoots	Small Shoots	Shoots	Leafing
Roots	No Roots	Pre-Roots	Small Roots	Roots	Branching

Table 3.4.3

Comparative responses of clone AE2 callus to media containing different growth regulators (mg l^{-1}) at 35 days. The results at 14, 21 and 28 days are shown in Table 19 of the Appendix.

		6-BAP						Kinetin			
		0.0		1.0		2.0		1.0		2.0	
IAA	NAA										
0.0	0.0	2	2	1	4	2	3	1	2	1	3
		1	1	1	2	1	1	1	2	1	1
1.0	0.0	2	4	2	4	1	2	2	2	1	3
		1	1	1	1	1	1	1	1	1	1
0.0	1.0	5	2	5	3	3	1	2	2	1	3
		1	1	1	1	1	1	1	1	1	1

Scale

G	C
S	R

G=Growth C=Colour

S=Shoots R=Roots

3.4.2 Regeneration of stem and lamina callus

With the exception of NAA the growth regulators of the previous experiment were used in attempts to regenerate callus initiated from stem and leaf lamina excised from a 3-month old plant.

3.4.2.1 Experimental procedure

Excised stem pieces (*ca.* 1cm. long) and lamina discs (*ca.* 1cm. diam.) were taken from the upper region of a 3-month old plant (cv. Cayenne) and sterilized before being placed onto various regeneration media. The media, contained in 9cm. diam. Petri dishes, consisted of 20ml. SH medium with added growth regulators the type and concentration of which are shown in Table 3.4.1. The stem and lamina pieces were maintained in culture for 35 days and the growth, colour and extent of regeneration (shooting and/or rooting) of the initiated callus was scored after 14 days, and subsequently after every 7 days, using the arbitrary scale shown in Table 3.4.2.

3.4.2.2 Effect of growth regulators on stem or lamina callus

It can be seen from scores at 35 days presented in Table 3.4.4 a. that although regeneration did not take place there were differences in the growth and colour of the stem callus which emerged over the course of the experiment. Irrespective of treatment the callus was moderate to fast growing with yellow or green cells, although the callus on media containing 6-BAP produced more green cells (Table 3.4.4 a.). There were no differences in colour or growth associated with increasing IAA in conjunction with either cytokinin (Table 3.4.4 a.). Early signs of root regeneration were found from stem callus on medium with 2.0mg l^{-1} 6-BAP and 1.0mg l^{-1} IAA, and also 2.0mg l^{-1} kinetin either alone or with 1.0mg l^{-1} IAA (Table 3.4.4 a.).

It can be seen from the scores at 35 days presented in Table 3.4.4 b. that although regeneration did not take place there were differences in the growth and colour of lamina callus which had emerged over the course of the experiment. Media containing 6-BAP supported slow to moderate growth and green cells, although callus on 2.0mg l^{-1} 6-BAP with either of the two highest IAA concentrations was slow growing with white or brown cells (Table 3.4.4 b.).

Table 3.4.4

Comparative responses of (a). stem and (b). lamina derived callus to media containing different growth regulators (mg l^{-1}) at 35 days. The results at 14, 21 and 28 days are shown in Tables 20, 21 and 22 of the Appendix

(a). Stem

		IAA 0.0		IAA 1.0		IAA 2.0		IAA 5.0		Control	
6-BAP 1.0	Kinetin 1.0	3	4	3	3	3	4	3	4	1	1
		1	1	1	1	1	1	1	1	1	1
6-BAP 2.0	Kinetin 1.0	3	2	3	5	3	5	4	4		
		1	1	1	1	1	1	1	1		
6-BAP 1.0	Kinetin 2.0	3	3	4	4	4	4	3	4		
		1	1	1	1	1	1	1	1		
6-BAP 2.0	Kinetin 2.0	3	4	3	3	3	4	3	2		
		1	2	1	2	1	1	1	1		

(b). Lamina

		IAA 0.0		IAA 1.0		IAA 2.0		IAA 5.0		Control	
6-BAP 1.0	Kinetin 1.0	2	2	2	2	2	2	3	4	1	2
		1	1	1	2	1	2	1	3	1	1
6-BAP 2.0	Kinetin 1.0	3	4	3	3	1	2	2	1		
		1	3	1	1	1	1	1	1		
6-BAP 1.0	Kinetin 2.0	3	3	2	2	2	2	2	2		
		1	1	1	2	1	1	1	1		
6-BAP 2.0	Kinetin 2.0	2	3	1	2	1	3	1	3		
		1	1	1	1	1	1	1	1		

Scale

G	C
S	R

G=Growth C=Colour

S=Shoots R=Roots

There was slow growth or no growth in the kinetin supplemented callus especially with the two highest IAA concentrations, while cells were predominantly white with some yellow cells (Table 3.4.4 b.). It can be seen in Table 3.4.4 b. that signs of root regeneration were found in callus with low 6-BAP and in all but one of the IAA concentrations. In this respect, callus with the highest IAA concentration produced roots after 35 days from green, slow to moderately growing callus (Table 3.4.4 b.). Early signs of root regeneration were also found in green callus on medium supplemented with 2.0mg l^{-1} 6-BAP alone (Table 3.4.4 b.).

3.4.3 Regeneration of hypocotyl callus

With the exception of kinetin, and the addition of zeatin, the growth regulators of the last experiment were used in attempts to regenerate from callus derived from hypocotyl tissue.

3.4.3.1 Experimental procedure

Hypocotyls were excised from two-week old germinated seedlings and cut into pieces (*ca.* 1cm. long) in a sterile Petri dish. The pieces were placed on various regeneration media in 3cm. Petri dishes, which consisted of 15ml. SH medium with growth regulators the types and concentrations of which are presented in Table 3.4.1. These were maintained for 35 days and the growth, colour and extent of regeneration (shooting and/or rooting) was scored after 14 days, and subsequently after every 7 days, using the arbitrary scale shown in Table 3.4.2.

3.4.3.2 Effect of growth regulators on hypocotyl callus

The scores after 35 days show that as well as causing differences in growth and colour the growth regulators were successful in regenerating hypocotyl callus (Table 3.4.5). All treatments supported callus with slow to moderate growth and many coloured cells, while in some the appearance of different stages of root and shoot regeneration was observed (Table 3.4.5). An increase in IAA with either 6-BAP or zeatin induced root regeneration particularly when used alone or with low cytokinin, the use of the highest IAA concentration without cytokinin induced roots from yellow or green callus (Table 3.4.5).

Table 3.4.5

Comparative responses of hypocotyl derived callus to media containing different growth regulators (mg l^{-1}) at 35 days. The results at 14, 21 and 28 days are shown in Tables 23, 24 and 25 of the Appendix

6-BAP

IAA \ 6-BAP	0.0	1.0	2.0	5.0	8.0	10.0	12.0
0.0	1 1 1 1	1 1 1 1	1 2 5 2	1 1 5 2	1 1 5 2	1 2 3 3	1 2 2 1
1.0	2 2 1 1	2 2 1 1	3 2 1 1	3 3 2 1	1 3 4 1	3 3 3 1	3 2 2 1
5.0	2 2 1 2	3 3 1 2	3 3 2 2	3 3 2 2	2 3 2 2	2 2 3 2	2 3 3 3
10.0	3 4 1 4	3 3 2 2	3 2 3 1	3 3 2 3	3 2 3 1	2 2 2 3	3 2 1 3

Zeatin

IAA \ Zeatin	0.0	1.0	2.0	5.0	8.0	10.0	12.0
0.0	2 1 1 1	2 1 1 1	1 3 5 1	1 3 4 1	1 3 4 2	1 2 4 1	1 2 3 3
1.0	1 1 1 1	2 2 1 3	2 2 2 3	2 2 3 3	2 2 3 2	2 3 3 2	3 3 3 2
5.0	1 2 2 3	2 2 3 1	3 2 3 2	3 4 3 2	3 3 4 2	2 2 3 2	2 2 4 1
10.0	3 3 1 3	2 2 4 4	2 4 3 2	2 4 3 2	3 4 5 2	2 4 3 2	2 4 4 2

Scale

G	C
S	R

G=Growth C=Colour

S=Shoots R=Roots

An increase in either the 6-BAP or zeatin concentration caused more shoots to arise from the callus, however, in the absence of IAA there was little callus growth and the regeneration found in these treatments appeared to be from the explant and not the callus (Table 3.4.5, Fig 3.4.1).

The use of 2.0, 5.0, 8.0 and 10.0mg l^{-1} 6-BAP with 10.0mg l^{-1} IAA and, to a lesser degree, 5mg l^{-1} IAA caused the formation of yellow callus from which small roots and shoots were formed (Table 3.4.5). Media containing zeatin supported the more advanced stages of regeneration, here the full concentration range of zeatin with 1.0, 5.0 and especially 10mg l^{-1} IAA produced small to fully established roots and shoots (Table 3.4.5, Fig 3.4.2). In particular, leafy shoots were found originating from green callus on medium containing 8.0mg l^{-1} zeatin and 10.0mg l^{-1} IAA (Fig 3.4.2). Advanced rooting and shooting was also found in callus on medium containing 1.0mg l^{-1} zeatin and 10.0mg l^{-1} IAA (Table 3.4.5).

3.4.4 Summary of the regeneration of callus material

In the first experiment regeneration using callus material of clone AE2 was not successful. It was found that NAA stimulated cell growth rather than causing it to slow down. In the next experiment, IAA and 6-BAP induced some regeneration from slow growing, green callus of stem and particularly lamina callus. In contrast, callus derived from hypocotyl regenerated readily on media with a combination of auxin and cytokinin, particulary IAA and zeatin. It appeared that the auxin induced greater rooting while the cytokinin caused shooting. The auxin was also responsible for inducing callus growth. Furthermore, the use of cytokinin alone also induced regeneration directly from the explant. These results show that callus derived from a young explant such as hypocotyl is more ready to regenerate in response to the appropriate regeneration conditions, and the failure to regenerate callus of clone AE2 appeared to be a result of the material and not the growth regulators used.

Figure 3.4.1

Root and shoot regeneration direct from the hypocotyl explant on media containing cytokinin only.

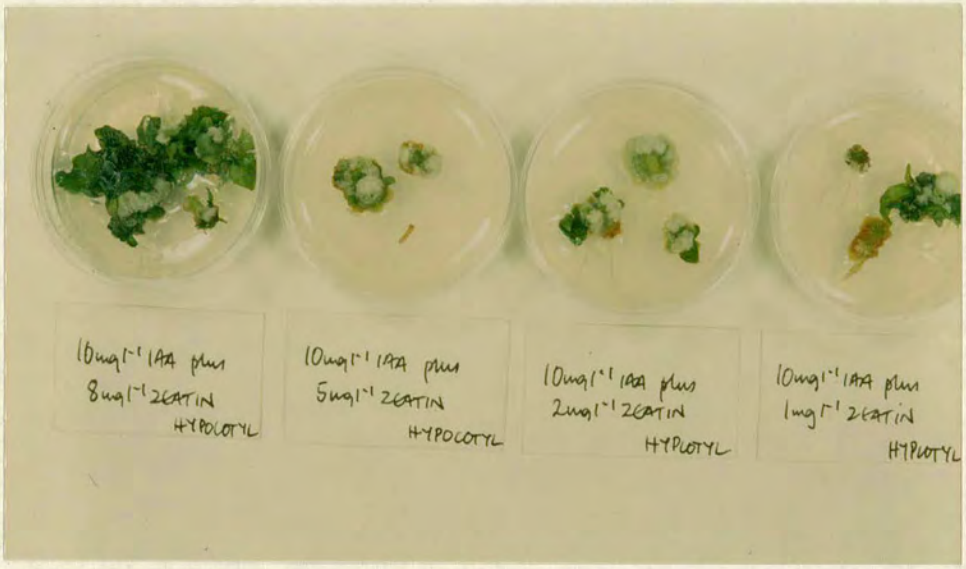
Root

Shoot

Control

Figure 3.4.2

Root and shoot regeneration from hypocotyl derived callus on media containing 10.0 mg l^{-1} IAA with different concentrations of zeatin.



CHAPTER FOUR

DISCUSSION

Induction and establishment of callus tissue from the explant

The establishment of callus from an explant is accompanied by changes in the genetical, metabolic and physiological characteristics of the cells and leads to the development of a heterogeneous culture (Yeoman and Forche 1980). However, there is little evidence to support the premise that explants from different parts of an individual plant give rise to cultures that are different from one another (Haddon and Northcote 1976). This is also supported by the results in this thesis which describe the effect of the position of the explant within an individual on the relative growth rate (RGR) of resultant callus cultures. Here it was shown that there were no differences in RGR among cultures derived from replicate pieces of stem from the upper region of the plant, or among cultures from stem, petiole or lamina from the upper or lower regions during a prolonged period. Furthermore, there were no differences in RGR between callus of stem and petiole from the upper and lower regions, whereas the differences between the upper and lower lamina derived cultures were due only to the slow growth of callus from the upper region over the first four weeks of the experiment. Interestingly, Wernicke and Brettell (1980) have shown that explants from the base of a young leaf of *Sorghum bicolor* formed proliferating cultures whereas those from older regions of the leaf did not, suggesting that younger material is more amenable for callus induction. However, this report did not consider the influence of explants from within an individual on the long term growth or relative growth rate of cultures. In this respect, the evidence available although fragmentary does not support the proposition that explant position within an individual affects the relative growth rate of callus cultures.

However, there is in the literature evidence to suggest that different parts of an individual plant can give rise to cultures which display differences in secondary metabolism in, for example, isozyme patterns (Arnison and Boll 1975), amounts of essential oils (Nagel and Reinhard 1975) and levels of lignanes (Kadkade 1982). In contrast, Speake *et al.* (1964) could not detect any differences in the amounts of nicotine among callus cultures derived from root, stem or leaf, and Dhoot and Henshaw (1977) reported that callus cultures initiated from different parts of *Hyoscyamus niger* seedlings accumulated similar levels of alkaloids.

Further results presented in this thesis show that similarities in the growth of cultures of different origins are not only restricted to parts of the same plant, for example the RGR of stem callus derived from each of three individual plants within a group was similar. It should be recognised that the plant used in the comparison, *Capsicum frutescens* cv. Cayenne, is an inbreeding species, therefore it is likely that the three plants were reasonably homozygous (Purseglove 1968). Zenk *et al.* (1977) showed a positive correlation between high and low yielding plants derived from a group of 'homozygous' seeds of *Catharanthus roseus* and the amount of secondary product formed in culture. It would seem therefore that differences do exist among plants from a homozygous population when comparisons are made of some characteristics, such as the biosynthesis of a secondary product.

This similarity in RGR among callus cultures derived from individual plants of *Capsicum frutescens* cv. Cayenne was also found among plants taken from different age groups. Contrary to this, Ketel *et al.* (1985) found that tissue from older plants produced cultures with higher growth rates although this study only extended over the first three subcultures. King (1980) has made the obvious suggestion that the most rapidly growing callus tissue is derived from the youngest and most active parts of a plant. Indeed, it might be expected that stem explants taken from the youngest regions of *Capsicum* would have been in a similar developmental state in plants of different ages, and would have all responded rapidly in culture.

In line with the general view from previous published work it was shown in this study that the use of different *Capsicum* 'species' as a source of explanted material produced differences in the appearance and RGR of the resultant cultures. Two of the species tested, *Capsicum baccatum* and *Capsicum frutescens*, were easier to put into culture than the other three species which responded very poorly producing slow growing, brown and aggregated callus which deteriorated with time in culture. However, these results were obtained using a medium prepared specifically for the culture of *Capsicum frutescens*. Differences in culture growth are also found in other species, and varieties of other species. In rice, for example, Mikami and Kinoshita (1988) have shown differences in the fresh weight of cultures derived from 108 rice varieties, whereas Mok and Mok (1977) showed differences in the fresh weight of callus derived from seven bean species. However, the genetic differences among plants within a species or variety must be taken into

consideration when comparisons such as these are made. In this thesis all the 'species' used, apart from *Capsicum frutescens* cv. Cayenne, were outbreeders which suggests that because of the among-plant variation within these plant groups it is likely that the selection of a single individual would have been unrepresentative of the group.

One other important result to emerge from these studies was that the RGR of nearly all the callus cultures, irrespective of explant origin, fell consistently with increasing time in culture. When compared to the overall results found from the investigations on explant origin, it would appear that the culture conditions were having a greater affect on callus culture than the differences derived from the explant material. Indeed, other evidence indicates that although some variation may have originated from the explant, the greater part occurs during the tissue culture period itself (Larkin and Scowcroft 1981, Lorz and Scowcroft 1983).

The expression of variation during tissue culture

Irrespective of origin, a heterogeneous cell population will develop from an explant placed in culture (Yeoman and Forche 1980). As it has been already stated some of this heterogeneity may be from the explant while most will develop in culture giving rise to complex intercellular variation (Hall and Yeoman 1987). By initiating callus the close intercellular relationships intrinsic to the explant tissue are removed and epigenetic and genetic stability of the component cells are impaired (Yeoman 1987). Although variation is revealed in the morphological and metabolic characteristics of the culture the underlying cause of these differences must be due to specific changes, permanent or not, in the genetic complement of the cells (Bayliss 1980, Gould 1986, Shirzadegan *et al.* 1989).

Although phenotypic variation is relatively simple to identify and record, the identification and assessment of the underlying genetic changes is difficult or near impossible to locate. Measurements of chromosome number and ploidy level in cells of a culture have been carried out using cell sorting and microdensitometric techniques (Brown 1984, Ramulu and Dijkhuis 1986, Pijnacken *et al.* 1989).

More recently patterns of alteration in the mitochondrial DNA of cultured cells in both dicotyledonous and monocotyledonous species have been

investigated by restriction enzyme analysis (Chowdhury *et al.* 1988, Shirzadegan *et al.* 1989). The more conventional method of fixing and staining to determine chromosome number provides more accurate data but only for cells in mitosis, as this constitutes only about 1% of the whole cell population at one time it is impossible to make an assessment of the whole population (Orton 1983).

However, a measure of intercellular variation as expressed by the differences that exist in cell phenotype, in particular the amounts of coloured secondary products, can be made (Sato and Yamada 1984, Hall and Yeoman 1986,1987). Microspectrophotometric analysis of the intracellular concentration of rosmarinic acid in individual cells of an *Anchusa* culture has revealed great differences within the population (Chaprin and Ellis 1984). Similarly, microdensitometric determinations of individual cells in a *Catharanthus* culture have also shown a great range of values in anthocyanin content (Hall and Yeoman 1986).

The underlying causes of the differences in secondary metabolite yield among cells of a culture are not fully understood. However, Hall and Yeoman (1987) showed by cloning cells of a heterogeneous *Catharanthus* culture that the non-producing individuals could produce cultures that were capable of anthocyanin synthesis. This suggests that the differences were a result of an epigenetic rather than a genetic change, as it appeared that the incapacity of cells to produce anthocyanin was not permanent. Furthermore, Hall and Yeoman (1987) proposed that the discrimination between the non-productive and productive cell types was governed by a 'switching on' mechanism, involved in the anthocyanin metabolic pathway, which might be controlled by the interaction between the two cell types in the population. Although the nature of this interaction has not yet been fully investigated, it seems likely that the intercellular heterogeneity in secondary product synthesis of a culture is strongly affected by the interaction among individual cells and between the cells and the culture environment.

The technique of single cell cloning has also been used in attempts to assess intercellular variation within cultures (for review see Dougall 1987). In a series of experiments in this thesis it was shown that single cells isolated from a suspension and cultured individually gave rise to callus clones which exhibited considerable differences in appearance, colour, RGR and TCC. Over an eighteen month period some callus clones remained yellow-green and

friable with a high RGR, whereas others were slow growing, brown and aggregated, in this latter case the appearance of these characteristics preceded a rapid loss in cell viability and these clones eventually died. In a more detailed comparison on five of the clones in this study it became apparent that the differences among them was due to the differential deterioration of the culture characteristics examined. In this respect, all the changes were in the same direction as the RGR, TCC and SPCs always dropped whereas there was an increase in the browning and aggregation in all but one of the five clones under examination.

Differences in the growth, colour and morphology of cultures derived from single cells or small aggregates derived from a single cell have long been recognised as a characteristic of tissue cultures (Blakely and Steward 1964, Sievert and Hildebrandt 1965). Selby and Collin (1976) have studied cultures of twenty subclones of three onion varieties and these showed considerable differences in growth rate, friability, sliminess and pigmentation. Similar reports have been reported by Davey *et al.* (1971) who showed that three clones of *Atropa belladonna* differed in growth rate, morphology and chlorophyll content. From the cloning study in this thesis and the other evidence it is clear that clones from a culture will reflect intercellular variation within the original population, always remembering that changes arising during the cloning procedure will modify this pre-existing variation (Petiard *et al.* 1986). It is also likely that the differential deterioration observed in most of the pepper clones is related to the genetic composition of the cloned cells in the original population. From this it would follow that clones which remained stable over the eighteen month period were probably derived from parent cells with a more stable genome.

The pre-existing variation within a cultured cell population may be exploited by cloning particularly in the establishment of cultures with increased biosynthetic activity (Curtin 1983, Tabata and Fujita 1985, Dix 1986). Zenk *et al.* (1977) have shown in cloning experiments with cells of *Catharanthus roseus* that cultures can be produced which exhibit differences in indole alkaloid content including some which produced higher amounts of alkaloid. Differences in the nicotine content among cultures of tobacco resulting from cloning experiments have also been demonstrated (Tabata and Hiraoka 1976, Ogino *et al.* 1978). Ellis (1985) has shown that suspension cultures of *Anchusa officinalis* established from single cells produced different amounts of

rosmarinic acid. Hall and Yeoman (1987) have also reported that the anthocyanin content of twenty six clones established from a culture of *Catharanthus roseus* was found to vary 30 fold. Tremouillaux-Guiller *et al.*, (1988) have also shown considerable variability in the dihydrofuroquinoline content among clones of *Choisya ternata*.

In this study it has been shown that pepper clones lost the ability to produce phenolic compounds with increasing subculture. This result is consistent with the general experience that cells selected for their high yields of secondary products lose the ability to accumulate these substances with time in culture, and that a continuous programme of recloning is necessary to retain the high yielding properties of the culture (Ogino *et al.* 1978, Deus-Neumann and Zenk 1984).

The phenotypic differences which have been regularly observed among clones in this study are not necessarily restricted to the genetic differences among the cells from which the clones were established. Both the cloning procedure and the culture conditions to which the clones were exposed are likely to have affected the resulting variation. Nevertheless, the results obtained are consistent with the view that cultural factors will increase the pre-existing variation by, for example, accelerating the deterioration in morphology, growth and metabolism of the clones. One clone, AE2, established from the original parent culture maintained a marked degree of morphological and metabolic stability and outlived the others. The reasons for the stability of AE2 are not completely understood. However, one possible explanation for the emergence of a stable clone would be linked to its origins.

It is not unreasonable to suppose that this clone was derived from a genetically stable cell, and because of this was unlikely to have experienced any gross genetic changes over the initial period of clone establishment. This would result in a relatively uniform population of genetically similar cells. Presumably, these viable cells would proliferate and outgrow any aberrant individuals and therefore dominate a culture retaining its genetic integrity and remaining predominant in the callus culture over a series of subcultures.

Variation within clone AE2 and its manipulation in different culture regimes

The nature of the uniformity and prolonged stability of clone AE2 in culture was also investigated in this study. Replicate measurements of TCC and TPC of different samples from each of three callus cultures of the clone at any one time showed that, although there were no differences in TPC there were in TCC among the randomly chosen samples. This variation in the concentration of the primary photosynthetic pigment within a cell population is not uncommon, as has been shown when only a proportion of cells within a culture actually accumulate chlorophyll when exposed to light (Dalton and Peel 1983). However, further work showed that there were no clear differences in appearance, RGR, TPC or even TCC among the daughter cultures derived from this clone in callus, suspension or immobilized suspension regimes over twelve weeks. Any differences that did occur were restricted to the production of SPCs and were sporadic and inconsistent in nature. Apart from the phenolic patterns these results showed that differences did not exist, even in the chlorophyll content, among samples taken from the parent over a series of subcultures even though some of the material had been transferred to another culture condition. This would support the premise (see above) that clone AE2 was made up of a relatively homogeneous population of cells. However, further results showed that there was a significant drop in the TCC, and the proportion of green cells, within the individual daughter cultures over this period which was not linked to the culture conditions. The reason for this reduction is not fully understood. However, the low chlorophyll content could explain why the differences were found among the samples taken directly from the parent and not among the resultant daughter cultures.

After this twelve week period it was found that although the callus daughter cultures became increasingly variegated and revealed inconsistent phenolic patterns, there were generally no differences found among or within the cultures in the other characteristics measured over prolonged subculture. However, this stability was not a feature of the cultures maintained as suspensions or immobilized suspensions. After twelve weeks, differences in all the characteristics measured were found both within and among cultures in these two culture conditions. Although it had not been noticed earlier in the experiment these differences appeared to have originated from each of the three suspension cultures derived after the subculture of the parent. In this

respect there were particular differences associated with each group of daughter cultures derived from the four week old parents, for example, one parent gave rise to cultures which showed a sharp decline in RGR, TCC, TPC and SPCs and a noticeable increase in browning and aggregation after twelve weeks.

These results would indicate that the callus condition provided a more stable environment for the daughter cultures. Indeed, this is demonstrated by the stability of clone AE2 over a prolonged period in culture prior to the study. In this respect, the callus presumably established a state of equilibrium with the conditions over successive subcultures. Furthermore, it has been suggested that the high cell-to-cell contact present in callus would permit chemical and physical gradients to exist among cells, thereby creating a more organised and stable condition in some ways similar to the intact plant (Yeoman 1987). In this respect, the transfer of callus to liquid medium would have disturbed the organisation of the cells making them more dispersed and isolated within the environment. Once free from the organised state, single cells or small cell aggregates within the suspension culture would respond in different ways to environmental stress. This would inevitably result in the emergence of variant and unstable cell types in this culture condition. The possible effects of medium constituents on the generation of variation in culture is discussed in the next section.

Some of these suspension and immobilized suspension cultures produced a large number and amount of unknown and known phenolics. The fact that the callus cultures were less active in this experiment was unusual as the greater cell to cell contact is known to favour secondary metabolism (Lindsey and Yeoman 1984a). However, the lower number of SPCs in callus culture may be related to the high RGR and friability observed in this regime, while the high activity in some suspension cultures might have resulted from the increased aggregation. It was not suprising that immobilized cultures produced many known SPCs, including vanillin, cinnamic acid, ferulic acid, coumaric acid and capsaicin, as this procedure stimulates secondary metabolism in cell cultures. Elsewhere in this laboratory, cells of *Capsicum frutescens* immobilized in reticulate polyurethane foam have been found to produce considerably higher amounts of capsaicin than suspension cultures (Yeoman *et al.* 1980, Lindsey and Yeoman 1984a, Lindsey and Yeoman 1984b).

The reason for the inconsistent SPC patterns in all three culture conditions would appear to be a result of epigenetic rather than genetic change. The characteristic disappearance of the known and unknown phenolics at one subculture then followed by their reappearance at a later subculture would support the reversible nature of these epigenetic changes. It is therefore possible that the expression of genes involved in phenolic synthesis is temporarily lost and can be recovered at a later stage in culture. In support of this view, Dougall *et al.* (1980) indicated that changes in the ability of carrot cells to accumulate anthocyanins involved no qualitative change in the DNA content, and Mok *et al.* (1976) similarly could not confirm that mutation in cultured carrot cells was a cause of altered pigmentation.

Effects of medium constituents on the generation of variation in suspension cultures

The pattern of growth and development of cells within a culture is a product of the interaction between the genome of the cells and the culture conditions (Lindsey and Yeoman 1985). Indeed the heterogeneity found within a culture is a result of the differences in the response of individual cells to the environment. This heterogeneity is made more complex by the fact that the culture environment is not stable as the components within it fluctuate (Knobloch and Berlin 1983, Lindsey 1985, Gould 1986).

Cell cultures require a balance of components in the medium for successful growth. Such a medium usually contains sucrose, as a carbon source, a mixture of inorganic constituents, including nitrate and phosphate, together with growth regulators all adjusted to a suitable pH (Dougall 1980). As individual cells may differ in their requirements for these components a culture will be made up of a mixture of cell groups that show, for example, different growth patterns in response to the culture medium. The nature of these differential requirements has not yet been fully investigated, however, some reports suggest that these differences are intrinsic to the explant from which cultures were derived. Torrey (1965) found that explants from the roots of peas contained two distinct populations (diploid and tetraploid) of cells which differed in their growth requirements, and Snijman *et al.* (1977) have also shown that the variability in the growth of cells in cultured tobacco pith explants could be related to differences in the endogenous levels of growth regulators.

Indeed cultured cells have been shown to differ in their requirements for growth regulators in the medium (for review see Mantell and Smith 1983). Changing the concentration of, the auxin, 2,4-D caused different growth responses in cultures of Paul's Scarlet Rose (Davies 1972) and in *Haplopappus gracilis* (Constabel *et al.* 1971). In contrast, the results in this thesis show that neither the RGR or TCC of suspension cultures of clone AE2 was affected by changes in the 2,4-D concentration. It is probable that this clonal material contained groups of cells that differed in their requirements for auxin, and would proliferate preferentially according to the level of auxin in the medium. In addition it would appear that some of the cells present were capable of growth in a medium without 2,4-D, suggesting that they were auxin habituated. Alternatively, it is possible that the whole culture was habituated to 2,4-D which could also explain the indifferent response to changes in the concentration of this growth regulator.

Results from this study have also shown that the morphological and metabolic characteristics of the suspension cultures were not altered by changes in the initial pH of the medium. It is perhaps less likely that this indifference resulted from the presence of pH 'tolerant' cell types as cultures of many plant species are capable of growing successfully within a pH range from 5 to 7.

Unlike the results of studies in which 2,4-D and pH were varied it has been shown that reductions in the concentrations of sucrose, nitrate and phosphate normally used to sustain growth resulted in a marked drop in the RGR and TCC, with an increase in the number of brown and aggregated cells. Furthermore, higher concentrations of the nutrients normally used appeared to benefit the growth of the cultures, which indicates that changes in these constituents had a greater effect on the culture characteristics than either 2,4-D or pH. This sensitivity to changes in the nitrate and phosphate levels in the medium have also been observed in *Capsicum frutescens* (Lindsey 1985), as well as in sycamore (Carceller *et al.* 1971) and periwinkle cultures (Knobloch and Berlin 1980). Gould *et al.* (1981) have shown that sycamore cells starved of nitrogen are unable to initiate DNA synthesis and become blocked in the G_1 phase of the cell cycle, while phosphate-deficient cultures contain a significant fraction of cells blocked in G_2 which are unable to enter mitosis. It has also been shown that carbon limitation in cultures accelerates entry into the stationary phase, and these cells suffer a decline in viability (Gould *et al.* 1981).

Factors involved in the control and production of secondary products in cell cultures appear to be sensitive to changes in medium components (Mantell and Smith 1983). It is not suprising, therefore, that phenylalanine ammonia lyase (PAL), a key regulatory enzyme involved in the phenylpropanoid pathway in plants, is particularly sensitive to such changes (Jones 1984). Bevan and Northcote (1979) found that a decrease in the amount of auxin supplied and an increase in the cytokinin concentration of the medium reduced growth and increased PAL activity in bean cultures. Westcott and Henshaw (1976) also found that PAL activity increased considerably following nitrate depletion in suspension cultures of sycamore, while a similar response was observed after phosphate limitation in suspension cultures of *Nicotiana tabacum* (Knobloch and Berlin 1981). In this study, the differences in PAL activity observed were either caused by changes that were intrinsic to a particular culture or group of cultures within a treatment, or corresponded to changes in the morphological state of the cultures. In the latter case, PAL activity was significantly higher in cultures that were generally slow growing, green or brown and aggregated, a condition caused by nutrient depletion.

The reduced amount of growth and protein synthesis brought about by these limiting growth conditions is likely to have increased the availabilty of phenylalanine to secondary metabolism. Indeed, there is evidence that cell growth rate directly regulates secondary metabolism by affecting the kinetic partitioning of precursors between primary and secondary metabolic pathways (Yeoman *et al.* 1982a, Yeoman 1987). In this respect, Lindsey (1985) has shown that under nitrate limiting conditions the incorporation of radioactive ^{14}C phenylalanine into protein is reduced, while the incorporation into capsaicin correspondingly increases in immobilized chilli pepper cells. It is debatable whether the high PAL activity found in these studies with clone AE2, under nutrient limiting conditions, was caused by an increased availability of phenylalanine. It would seem more likely that PAL increased in response to the stress condition independently and not due to the increased availability of the substrate.

Regeneration of callus cultures

A characteristic of some callus cultures is the ability to regenerate after a prolonged period in culture. However, in order to regenerate even from totipotent cultures, a special balance of growth regulators is required (Flick *et*

al, 1983). Under such conditions the variation present in the callus could be shown more clearly in the regenerated plant and even used to practical gain as a source of genetic diversity (Larkin and Scowcroft 1981, Ryan *et al.* 1987). This phenomenon, termed somaclonal variation, has been well documented in terms of its ubiquity, of its likely causes and of its potential impact on plant improvement (Scowcroft *et al.* 1985, Larkin *et al.* 1985, Karp and Bright 1985).

The results presented in this thesis have shown that callus of clone AE2 failed to regenerate in response to a variety of growth regulators. Although it is possible that the appropriate balance of growth regulators was not used, it is more probable that the callus had lost the ability to regenerate. This suggests that although many of the morphological and metabolic characteristics remained stable, as had been shown in previous experiments, the mechanisms involved in regeneration appeared more susceptible to change with prolonged culture. Indeed, it is a widely accepted fact that there is a progressive fall in the regenerative potential of cells with increasing time in culture (McCoy *et al.* 1982, Karp and Maddock 1984). Other reports suggest that the regeneration of pepper callus is difficult if not impossible to achieve, and the only evidence for regeneration has been directly from the explant such as cotyledon, hypocotyl, embryo and anther (Gunay and Rao 1978, Agrawal and Chandra 1983, Morrison *et al.* 1986).

Further results in these studies would support a drop of regenerative potential with time in culture, as newly initiated callus from lamina, stem and especially hypocotyl regenerated most easily. Callus material from hypocotyl responded quickly to a medium containing IAA and zeatin by producing both shoots and roots. It is well established that both auxin and cytokinin are required in the regeneration of many plant species from culture (Evans *et al.* 1981). It should be added that the combination of growth regulators used in the most successful regeneration treatments with hypocotyl callus in this study were different from those applied to the callus of clone AE2. To fully establish the existence of a drop in the regeneration potential in the callus of *Capsicum* it would be important to subject clone AE2 callus to these optimum conditions.

Variation in cell culture; control and exploitation

Establishing callus cultures from stem, lamina or petiole from either two, three, four or five month old plants of *Capsicum frutescens* cv. Cayenne does not affect the relative growth rate over a prolonged period in culture. However, differences are likely to exist when plants of an outbreeding species are used, like the *Capsicum* wild types, because of the genetic variation among individuals. In this respect consideration must be made over the choice of plant part or plant, however, alternatively the poor response of some species to culture could be improved by modifying the growth medium. Once established in culture such species could be exploited as a new source of genetic diversity with, for example, a higher production of capsaicin.

An agitated liquid medium unlike an agar surface provides an unstable environment for cultured cells. This condition increases the number of variant cell types within a culture most of which, as the cloning experiment in these studies have shown, experience a decline in viability with increasing subculture. However, the use of suspension culture is necessary for many applications in tissue culture, in particular, exploiting genetic manipulation or secondary metabolism, or even for providing a source of genetic diversity in culture. In this respect, the variation that exists in suspension culture should receive particular attention with regard to firstly its control then its exploitation.

Using cloning techniques, stable suspension cultures could be established if the following steps were carried out. Firstly, the cells of the suspension culture to be cloned should be derived from newly initiated callus material. Secondly, the cells of the suspension should be subjected to flow cytometry to isolate those which had not experienced any changes in DNA content with a view of isolating these more 'stable' cells for cloning. Finally, once established the callus clones should be returned immediately to suspension culture and after a series of subcultures, to let the cells adapt or develop an equilibrium with the conditions, during which the cells should be prevented from being exposed to any large environmental stresses eg; nutrient depletion, stable suspension clones might be found.

FUTURE WORK

Future investigations could involve a more detailed examination of intercellular heterogeneity within suspension cultures by focussing on the differences among cells at any one time and among plants regenerated from single cells.

To assess the variation among cells in culture would necessitate identifying then measuring cellular constituents within the individuals of a population. One way to do this is by using flow cytometry which, in conjunction with a variety of flourescent agents, could quantify the DNA content of individual cells within a suspension culture (Kruth 1982, Brown 1984, Bergounioux *et al.* 1988). This tool could be used to develop a means for the possible control of variation in culture. For example, cells with a normal DNA content could be selected and used for establishing suspensions with greater stability. Immunohistochemical techniques could be employed to localise and determine the concentrations of enzymatic proteins involved in secondary metabolism which would provide a means of examining biochemical differences among cells within a suspension culture. For example, the localisation of the PAL enzyme using antibodies specific to the protein has been carried out in parsley seedlings (Jahnen and Hahlbrock 1988a,b). Although it has not yet been carried out with cultured cells this could provide a method for determining intercellular differences *in vitro*.

Analysis of variation among single cells is limited not only by the analytical techniques available but also by the inactivity of a large part of the genome in cultured cells. Regenerating plants from single cells of a suspension culture enables an examination to be made of the whole of the functional part of the genome and any novel variation it may contain. For example, in the regenerated condition there is an opportunity to measure changes in morphological, physiological and reproductive characteristics. Furthermore, the regenerated plant provides the opportunity for a more detailed examination of genetic and epigenetic changes, not shown by DNA levels, and also the chance to employ a range of molecular techniques which could, for example, measure specific changes in, say, gene expression.

With these considerations in mind a scheme of future work can be summarised as follows:-

Flow Cytometry

Quantification of cellular DNA content would provide;

1. an assessment of variation in DNA content among cells of a suspension culture at any one time.
2. the means to isolate cells with a chosen DNA content prior to cloning and regeneration.

Immunohistochemical techniques

Isolation and quantification of PAL at the cellular level would provide;

1. an assessment of variation in the amount of this enzyme among cells of a suspension culture at any one time.
2. the means to isolate cells with a high PAL content prior to cloning and establishment of suspension cultures with a greater biosynthetic activity.

Regeneration

The regeneration of plants from single cells would provide;

1. an assessment of the frequency and nature of variation among cells of a suspension culture as shown by the differences among regenerated plants.
2. the opportunity to assess characteristics which are not revealed in single cells of a culture.
3. the opportunity to assess the genetic nature of culture variance by molecular techniques or Mendelian genetics.

APPENDIX

Table 1

The effect of clone, culture regime, subculture and interaction on the relative growth rate of callus cultures over sixteen weeks as shown by the variance ratio (VR) determined by analysis of variance.

SOURCE OF VARIATION	DF	SS	SS%	MS	VR	LSD
Clones	4	255.9	61.8	63.9	200.8 **	0.45
Regime	1	1.3	0.3	1.3	4.1	0.28
Subculture	3	68.6	16.6	22.9	71.8 **	0.34
Clones.Regime	4	39.9	9.6	9.9	31.3 **	0.43
Clones.Subculture	12	12.3	2.9	1.1	3.2 **	1.5
Regime.Subculture	3	1.4	0.3	0.5	1.5	
Clones.Regime.Subculture	12	9.5	2.3	0.8	2.5	
Residual	80	25.5	6.2	0.3		
Total	119	414.4	100.0	3.5		

** denotes VR is significant at the 1% level

Table 2

Results of analysis of variance comparing differences in the relative growth rate between regimes at four weeks, among and within regimes at eight weeks and among and within each regime from twelve to thirty-two weeks using the variance ratios (VR) as determined by analysis of variance.

SOURCE OF VARIATION	DF	SS	SS%	MS	VR	LSD
Week Four						
Regime	2	17.2	63.1	8.6	5.1	**1.2**
Residual	6	10.1	36.9	1.7		
Total	8	27.3	100	3.4		
Week Eight						
Regime	2	44.9	61.6	22.5	19.5 **	**1.3**
Culture	2	2.5	3.4	1.2	1.1	**2.5**
Regime.Culture	4	4.8	6.6	1.2	1.1	
Residual	18	20.7	28.4	1.2		
Total	26	73.0	100	2.9		
Weeks Twelve to Thirty-two						
Callus						
Subculture	5	286	42.1	57.3	21.9 **	**1.2**
Culture	8	36.4	5.3	4.5	1.7	**1.6**
Subculture.Culture	40	75.6	11.1	1.9	0.7	
Residual	108	282	41.5	2.6		
Total	161	680	100	4.2		
Suspension						
Subculture	5	735	49.7	147	56.7 **	**1.2**
Culture	8	289	19.6	36.1	13.9 **	**1.6**
Subculture.Culture	40	172	11.6	4.3	1.6	
Residual	108	280	18.9	2.6		
Total	161	1477	100	9.2		
Immobilized-Suspension						
Subculture	5	232	30.0	46.6	25.1 **	**1.0**
Culture	8	159	20.5	19.9	10.7 **	**1.4**
Subculture.Culture	40	183	23.7	4.6	2.5	
Residual	108	200	25.8	1.9		
Total	161	776	100	4.8		

** denotes VR is significant at the 1% level

Table 3

The effect of subculture, regime, culture and interactions on the relative growth rate in all cultures from twelve to thirty-two weeks as shown by the variance ratios (VR) determined by analysis of variance.

SOURCE OF VARIATION	DF	SS	SS%	MS	VR	LSD
Subculture	5	1008	23.3	201	85.6 **	
Regime	2	1395	32.2	6977	296 **	0.4
Culture	8	267	6.2	33.5	14.2 **	
Subculture.Regime	10	246	5.7	24.7	10.5 **	
Subculture.Culture	40	169	3.9	4.2	1.7	
Regime.Culture	16	216	5.0	13.6	5.8	
Subculture.Regime.Culture	80	263	6.1	3.3	1.4	
Residual	324	763	17.6	2.4		
Total	485	4330	100	8.9		

** denotes VR is significant at the 1% level

Table 4

Values obtained from a comparative t-test of the relative growth rate between parent and daughter cultures from four to eight weeks, and and from eight to twelve weeks in callus (C), suspension (S) and immobilized suspension (IS) regimes.

			C	S	IS
		(1)	0.96	0.67	
Week		(2)	1.25	0.40	
4 x 8		(3)	0.85	0.93	
		(11)	0.35	0.21	2.12
	(1) x	(12)	1.25	0.40	0.44
		(13)	2.10	0.84	1.84
		(21)	1.31	0.40	0.37
Week	(2) x	(22)	0.31	1.31	1.12
8 x 12		(23)	0.51	1.34	0.56
		(31)	0.36	1.98	0.94
	(3) x	(32)	0.05	1.08	0.61
		(33)	0.03	2.05	0.34

** denotes T-value is significant at the 1% level

Table 5

Results of analysis of variance comparing differences in the total chlorophyll content between regimes at four weeks, among and within regimes at eight weeks and among and within each regime from twelve to twenty-eight weeks using the variance ratios (VR) as determined by analysis of variance.

SOURCE OF VARIATION	DF	SS	SS%	MS	VR	LSD
Week Four						
Regime	2	57998	87.3	28999	20.6 **	**3.3**
Residual	6	8412	12.6	1402		
Total	8	66411	100.0	8301		
Week Eight						
Regime	2	32709	39.8	16355	7.2 **	**57.2**
Culture	2	4827	5.8	2414	1.1	**116**
Regime.Culture	4	3920	4.8	980	0.43	
Residual	18	4063	49.5	2257		
Total	26	82086	100.0	3157		
Weeks Twelve to Thirty-two						
Callus						
Subculture	4	7403	5.4	1850	2.4	**19.7**
Culture	8	14872	10.9	1859	2.4	**1.6**
Subculture.Culture	32	45964	33.6	1436	1.9	
Residual	90	68270	50.0	758		
Total	134	136510	100.0	1081		
Suspension						
Subculture	4	5352	20.8	1338	9.5 **	**8.5**
Culture	8	2948	11.5	368	2.6 **	**12.3**
Subculture.Culture	32	4747	18.5	148	1.1	
Residual	90	12663	49.2	140		
Total	134	25712	100.0	191		
Immobilized-Suspension						
Subculture	4	2056	22.6	514	12.4 **	**4.6**
Culture	8	667	7.4	83.4	2.0	**6.7**
Subculture.Culture	32	2615	28.8	81.6	1.9	
Residual	90	3739	41.2	41.6		
Total	134	9079	100.0	67.8		

** denotes VR is significant at the 1% level

Table 6

The effect of subculture, regime, culture and interactions on the total chlorophyll content in all cultures from twelve to twenty-eight weeks as shown by the variance ratios (VR) determined by analysis of variance.

SOURCE OF VARIATION	DF	SS	SS%	MS	VR	LSD
Subculture	4	4631	1.9	1157	3.6 **	
Regime	2	65499	27.6	32749	104 **	4.6
Culture	8	4369	1.9	546	1.7	
Subculture.Regime	8	10182	4.3	127	4.06 **	
Subculture.Culture	32	16252	6.8	507	1.6	
Regime.Culture	16	14119	5.9	882	2.8	
Subculture.Regime.Culture	64	37074	15.7	579	1.8	
Residual	270	84673	35.7	313		
Total	404	236802	100	586		

** denotes VR is significant at the 1% level

Table 7

Values obtained from a comparative t-test of the total chlorophyll content between parent and daughter cultures from four to eight weeks, and from eight to twelve weeks in callus (C), suspension (S) and immobilized suspension (IS) regimes.

			C	S	IS
		(1)	2.98 **	11.4 **	
Week		(2)	2.83 **	5.66 **	
4 x 8		(3)	3.80 **	13.6 **	
		(11)	1.91	0.10	0.71
	(1) x	(12)	1.70	0.20	1.66
		(13)	0.85	0.28	0.04
		(21)	1.83	1.29	1.72
Week	(2) x	(22)	2.13	1.37	0.49
8 x 12		(23)	1.78	0.92	1.53
		(31)	1.05	0.08	0.71
	(3) x	(32)	1.33	0.99	0.39
		(33)	0.39	0.54	0.82

** denotes VR or T-value is significant at the 1% level

Table 8

Results of analysis of variance comparing differences in the total protein content between regimes at four weeks, among and within regimes at eight weeks and among and within each regime from twelve to thirty-two weeks using the variance ratios (VR) as determined by analysis of variance.

SOURCE OF VARIATION	DF	SS	SS%	MS	VR	LSD
Week Four						
Regime	2	0.9	7.9	0.5	0.3	**3.2**
Residual	6	11.4	92.1	1.9		
Total	8	12.4	100	1.5		
Week Eight						
Regime	2	64.3	21.7	32.2	3.1 **	**3.9**
Culture	2	1.5	0.5	0.7	0.1	**7.9**
Regime.Culture	4	40.1	13.7	10.1	0.9	
Residual	18	189	64.1	10.5		
Total	26	295	100	11.4		
Weeks Twelve to Thirty-two						
Callus						
Subculture	5	1003	27.7	200	11.3 **	**3.2**
Culture	8	129	3.6	16.2	0.9	**4.4**
Subculture.Culture	40	552	15.3	13.8	0.7	
Residual	108	1929	53.3	17.8		
Total	108	3615	100	22.4		
Suspension						
Subculture	5	2974	26.1	594	12.6 **	**5.2**
Culture	8	1680	14.8	210	4.6 **	**7.1**
Subculture.Culture	40	1615	14.2	40.4	0.9	
Residual	108	5095	44.8	47.2		
Total	161	11366	100	70.6		
Immobilized-Suspension						
Subculture	5	2205	12.2	441	6.9 **	**6.1**
Culture	8	4068	22.5	508	8.1 **	**8.2**
Subculture.Culture	40	4932	27.3	123.3	1.9	
Residual	108	6842	37.9	63.4		
Total	161	18052	100	112		

** denotes VR is significant at the 1% level

Table 9

The effect of subculture, regime, culture and interactions on the total protein content in all cultures from twelve to thirty-two weeks as shown by the variance ratios (VR) determined by analysis of variance.

SOURCE OF VARIATION	DF	SS	SS%	MS	VR	LSD
Subculture	5	4606	13.7	921	21.5 **	
Regime	2	434	1.3	216	5.1 **	1.71
Culture	8	3438	10.3	429	10.1 **	
Subculture.Regime	10	1576	4.7	157	3.7	
Subculture.Culture	40	2961	8.9	74.0	1.7	
Regime.Culture	16	2441	7.3	152	3.6	
Subculture.Regime.Culture	80	4139	12.4	51.7	1.2	
Residual	324	13871	41.4	42.8		
Total	485	33467	100	69.0		

** denotes VR is significant at the 1% level

Table 10

Values obtained from a comparative t-test of the total protein content between parent and daughter cultures from four to eight weeks, and and from eight to twelve weeks in callus (C), suspension (S) and immobilized suspension (IS) regimes.

			C	S	ISI
		(1)	1.98	2.17	
Week		(2)	1.35	1.4	
4 x 8		(3)	1.85	1.93	
		(11)	2.09	1,83	2.94 **
	(1) x	(12)	1.36	2.45	0.56
		(13)	3.86 **	2.60	2.17
		(21)	1.46	1.02	0.65
Week	(2) x	(22)	1.49	0.71	1.65
8 x 12		(23)	0.05	0.94	0.73
		(31)	0.97	1.73	0.52
	(3) x	(32)	2.11	0.84	5.32 **
		(33)	1.98	1.85	0.75

** denotes VR or T-value is significant at the 1% level

Table 11

Results of analysis of variance comparing the effect of 2,4-D concentration and subculture on the relative growth rate (RGR), total chlorophyll content (TCC) and phenylalanine ammonia lyase activity (PAL) of cultures over sixteen weeks using the variance ratios (VR) as determined by analysis of variance.

SOURCE OF VARIATION	DF	SS	SS%	MS	VR	LSD
RGR						
Concentration	4	12.7	14.9	3.2	5.5 **	**0.85**
Subculture	3	12.4	14.5	4.1	7.2	**0.7**
Concentration.Subculture	12	36.8	43.3	3.1	5.3 **	**2.32**
Residual	40	23.0	27.1	0.5		
Total	59	84.9	100	1.4		
TCC						
Concentration	4	607	22.7	151	14.2 **	**0.75**
Subculture	3	931	34.8	310	29.0 **	**0.6**
Concentration.Subculture	12	707	26.5	58.9	5.5 **	**2.0**
Residual	40	428	16.0	10.7		
Total	59	2674	100	45.3		
PAL						
Concentration	4	79851	28.2	19963	7.6 **	**1.4**
Subculture	3	69933	24.7	23311	9.1 **	**1.32**
Concentration.Subculture	12	183.9	23.7	4.6	2.5	**4.9**
Residual	40	92647	32.8	2574		
Total	59	312148	100	5675		

** denotes VR is significant at the 1% level

Table 12

Results of analysis of variance comparing the effect of sucrose concentration and subculture on the relative growth rate (RGR), total chlorophyll content (TCC) and phenylalanine ammonia lyase activity (PAL) of cultures over sixteen weeks using the variance ratios (VR) as determined by analysis of variance.

SOURCE OF VARIATION	DF	SS	SS%	MS	VR	LSD
RGR						
Concentration	4	147	59.7	36.7	47.4 **	1.0
Subculture	3	37.2	15.1	12.4	16.0 **	0.83
Concentration.Subculture	12	30.9	12.6	2.6	3.3	2.7
Residual	40	31.0	12.6	0.7		
Total	59	246	100	4.2		
TCC						
Concentration	4	628	40.7	157	15.1 **	0.7
Subculture	3	197	12.7	65.7	6.3 **	0.62
Concentration.Subculture	12	299	19.4	24.9	2.4 **	1.98
Residual	40	415	26.9	10.4		
Total	59	1540	100	26.1		
PAL						
Concentration	4	1496	4.9	374	0.9	0.55
Subculture	3	2402	8.0	801	1.9	0.5
Concentration.Subculture	12	9888	32.9	824	2.0	1.55
Residual	40	16276	54.1	406		
Total	59	30065	100	509		

** denotes VR is significant at the 1% level

Table 13

Results of analysis of variance comparing the effect of nitrate concentration and subculture on the relative growth rate (RGR), total chlorophyll content (TCC) and phenylalanine ammonia lyase activity (PAL) of cultures over sixteen weeks using the variance ratios (VR) as determined by analysis of variance.

SOURCE OF VARIATION	DF	SS	SS%	MS	VR	LSD
RGR						
Concentration	4	155	66.5	38.9	199 **	**0.5**
Subculture	3	64.4	27.5	21.5	109 **	**0.42**
Concentration.Subculture	12	6.6	2.6	0.5	2.6	**1.32**
Residual	40	7.8	3.3	0.2		
Total	59	233	100	3.9		
TCC						
Concentration	4	3289	59.6	822	89.8 **	**0.7**
Subculture	3	254	4.6	84.9	9.3 **	**0.6**
Concentration.Subculture	12	1606	29.2	133	14.6 **	**1.85**
Residual	40	365	6.6	9.2		
Total	59	5516	100	93.5		
PAL						
Concentration	4	156699	71.2	39174	82.8 **	**0.6**
Subculture	3	28736	13.1	9578	20.2 **	**0.52**
Concentration.Subculture	12	15436	7.0	1286	2.7	**1.65**
Residual	40	18928	8.6	473		
Total	59	219800	100	3725		

** denotes VR is significant at the 1% level

Table 14

Results of analysis of variance comparing the effect of phosphate concentration and subculture on the relative growth rate (RGR), total chlorophyll content (TCC) and phenylalanine ammonia lyase activity (PAL) of cultures over sixteen weeks using the variance ratios (VR) as determined by analysis of variance.

SOURCE OF VARIATION	DF	SS	SS%	MS	VR	LSD
RGR						
Concentration	4	121	62.9	30.4	137 **	**1.52**
Subculture	3	23.5	12.2	7.8	35.3 **	**1.45**
Concentration.Subculture	12	39.3	20.3	3.3	14.7 **	**1.45**
Residual	40	8.8	4.6	0.22		
Total	59	193	100	3.2		
TCC						
Concentration	4	637	38.6	159	17.9 **	**0.7**
Subculture	3	84.0	5.1	28.0	3.2	**0.6**
Concentration.Subculture	12	573	34.7	47.7	5.3 **	**1.8**
Residual	40	355	21.5	8.8		
Total	59	1650	100	27.9		
PAL						
Concentration	4	202497	80.7	50624	336 **	**0.32**
Subculture	3	18846	7.5	6282	41.7 **	**0.28**
Concentration.Subculture	12	23485	9.3	1957	13.0 **	**0.95**
Residual	40	6021	2.4	150		
Total	59	250851	100	4251		

** denotes VR is significant at the 1% level

Table 15

Results of analysis of variance comparing the effect of pH and subculture on the relative growth rate (RGR), total chlorophyll content (TCC) and phenylalanine ammonia lyase activity (PAL) of cultures over sixteen weeks using the variance ratios (VR) as determined by analysis of variance.

SOURCE OF VARIATION	DF	SS	SS%	MS	VR	LSD
RGR						
Concentration	4	6.4	3.2	1.6	3.0	**0.85**
Subculture	3	165	82.3	55.1	103 **	**0.7**
Concentration.Subculture	12	8.4	4.2	0.6	1.3	**2.2**
Residual	40	21.3	10.6	0.53		
Total	59	201	100	3.4		
TCC						
Concentration	4	38.7	4.8	9.7	0.7	**0.85**
Subculture	3	40.3	4.9	13.5	0.9	**0.7**
Concentration.Subculture	12	182	22.5	15.2	1.1	**2.25**
Residual	40	546	67.6	13.6		
Total	59	808	100	13.7		
PAL						
Concentration	4	12080	11.7	3020	2.4	**1.0**
Subculture	3	27949	27.3	9316	7.4 **	**0.82**
Concentration.Subculture	12	11809	11.5	984	0.7	**2.7**
Residual	40	50659	49.4	1266		
Total	59	102497	100	1737		

** denotes VR is significant at the 1% level

Table 16

Results of analysis of variance comparing differences in the relative growth rate of stem, lamina and petiole derived callus cultures within each group from the upper region, and among groups within the upper and lower regions of a single plant using the variance ratios (VR) as determined by analysis of variance.

SOURCE OF VARIATION	DF	SS	SS%	MS	VR	LSD
Among upper stem						
Subculture	3	8.1	32.2	2.7	5.5	
Stem	2	3.1	12.0	1.5	3.1	**2.1**
Subculture.Stem	6	2.3	9.2	0.4		
Residual	24	11.7	46.5	0.4		
Total	35	25.2	100	0.7		
Among upper lamina						
Subculture	3	27.5	54.9	9.2	12.8 **	
Lamina	2	0.6	1.2	0.3	0.4	**0.9**
Subculture.Lamina	6	4.8	9.6	0.8	1.1	
Residual	24	17.1	34.3	0.7		
Total	35	50.0	100	1.4		
Among upper petiole						
Subculture	3	10.5	31.3	3.5	4.1	
Petiole	2	0.6	1.8	0.3	0.3	**0.9**
Subculture.Petiole	6	2.1	6.1	0.3	0.4	
Residual	24	20.5	60.8	0.8		
Total	35	33.6	100	0.9		
Among stem, lamina and petiole from the upper region						
Subculture	3	31.9	29.0	10.6	16.5 **	**1.1**
Explant	2	2.9	2.6	1.5	2.3	
Subculture.Explant	6	14.8	13.5	2.5	3.8	
Residual	94	60.7	55.1	0.6		
Total	105	110	100	1.1		
Among stem, lamina and petiole from the lower region						
Subculture	3	58.6	43.1	19.5	21.3 **	**1.3**
Explant	2	0.6	0.5	0.3	0.3	
Subculture.Explant	6	3.2	2.4	0.5	0.6	
Residual	88	80.1	59.1	0.9		
Total	99	143	105	1.4		

** denotes VR is significant at the 1% level

Table 17

Results of analysis of variance comparing differences in the relative growth rate of stem, lamina and petiole derived callus cultures between the upper and lower regions of a single plant using the variance ratios (VR) as determined by analysis of variance.

SOURCE OF VARIATION	DF	SS	SS%	MS	VR
Stem					
Subculture	3	25.4	36.4	8.5	13.2 **
Region	1	3.6	5.2	3.6	4.6
Subculture.Region	3	1.8	2.6	0.6	0.9
Residual	62	39.6	56.7	0.6	
Total	69	39.6	100	1.1	
Lamina					
Subculture	3	24.5	31.8	8.1	11.8 **
Region	1	6.7	8.7	6.7	9.8 **
Subculture.Region	3	17.1	22.2	5.6	8.3 **
Residual	57	39.1	51.1	0.6	
Total	64	87.3	113	1.4	
Petiole					
Subculture	3	38.1	37.3	12.7	12.8 **
Region	1	0.2	0.2	0.3	0.3
Subculture.Region	3	1.9	1.8	0.6	0.6
Residual	63	62.4	60.9	0.9	
Total	70	102	100	1.5	

** denotes VR is significant at the 1% level

Table 18

Results of analysis of variance comparing differences in the relative growth rate of stem derived callus cultures from plants within a group, of different age and genotype using the variance ratios (VR) as determined by analysis of variance.

SOURCE OF VARIATION	DF	SS	SS%	MS	VR	LSD
Plants in a group						
Subculture	3	49.6	30.8	16.5	18.7 **	**0.6**
Plant	2	2.2	1.3	1.1	1.2	**0.43**
Subculture.Plant	6	26.2	16.3	4.4	4.9	**1.4**
Residual	94	83.1	51.6	0.9		
Total	1.5	161.1	100	1.5		
Plants of different age						
Subculture	3	104	39.1	34.8	39.5 **	**0.55**
Age	3	5.8	2.2	1.9	2.2	**0.5**
Subculture.Age	9	55.3	20.7	6.1	6.9 **	**1.3**
Residual	125	110	41.2	0.9		
Total	140	275	103	1.9		
Plants of different genotype						
Subculture	3	55.6	18.3	18.5	30.9 **	**0.4**
Genotype	4	150	49.4	37.6	62.6 **	**0.5**
Subculture.Genotype	12	37.4	12.2	3.1	5.2 **	**1.2**
Residual	151	90.7	29.8	0.6		
Total	170	334	109	1.9		

** denotes VR is significant at the 1% level

Table 19

Comparative responses of clone AE2 callus to media containing different growth regulators (mg l^{-1}) at i). 14 days, ii). 21 days and iii) 28 days.

		6-BAP				Kinetin			
i).		0.0	1.0	2.0		1.0		2.0	
	0.0	1 1 1 1	1 2 1 2	1 3 1 1		1 2 1 2		1 1 1 1	
IAA	1.0	1 3 1 1	1 1 1 1	1 1 1 1		1 2 1 1		2 2 1 1	
NAA	1.0	3 4 1 1	5 3 1 1	3 2 1 1		1 2 1 1		1 2 1 1	
ii).		0.0	1.0	2.0		1.0		2.0	
	0.0	3 4 1 1	3 4 1 2	3 3 1 1		3 2 1 2		2 3 1 1	
IAA	1.0	3 4 1 1	3 4 1 1	3 2 1 1		3 4 1 1		3 2 1 1	
NAA	1.0	3 4 1 1	3 4 1 1	3 3 1 1		3 2 1 1		3 2 1 1	
iii).		0.0	1.0	2.0		1.0		2.0	
	0.0	3 2 1 1	1 3 1 2	2 3 1 1		3 2 1 2		2 3 1 1	
IAA	1.0	2 4 1 1	2 4 1 1	1 2 1 1		2 3 1 1		2 3 1 1	
NAA	1.0	5 3 1 1	5 3 1 1	4 3 1 1		2 2 1 1		2 2 1 1	

Table 20

Comparative responses of (a). stem and (b). lamina derived callus to media containing different growth regulators (mg l^{-1}) at 14 days.

(a). Stem

6-BAP \ IAA	0.0	1.0	2.0	5.0	Control
1.0	3 3 1 1	3 3 1 1	3 3 1 1	3 3 1 1	1 1 1 1
2.0	2 2 1 1	3 3 1 1	3 3 1 1	3 3 1 1	
Kinetin 1.0	3 3 1 1	3 3 1 1	3 3 1 1	3 3 1 1	
2.0	3 3 1 1	3 3 1 1	3 3 1 1	2 2 1 1	

(b). Lamina

6-BAP \ IAA	0.0	1.0	2.0	5.0	Control
1.0	2 2 1 1	2 2 1 1	2 2 1 1	2 2 1 1	1 1 1 1
2.0	3 3 1 3	3 2 1 1	1 2 1 1	2 1 1 1	
Kinetin 1.0	3 3 1 1	2 2 1 2	2 2 1 1	2 2 1 1	
2.0	2 2 1 1	1 2 1 1	1 2 1 1	1 2 1 1	

Scale

G	C
S	R

G=Growth C=Colour

S=Shoots R=Roots

Table 21

Comparative responses of (a). stem and (b). lamina derived callus to media containing different growth regulators (mg l^{-1}) at 21 days.

(a). Stem

6-BAP \ IAA	0.0	1.0	2.0	5.0	Control
1.0	3 3 1 1	3 3 1 1	3 3 1 1	3 3 1 1	1 1 1 1
2.0	2 2 1 1	3 4 1 1	3 3 1 1	3 3 1 1	
1.0	3 3 1 1	3 4 1 1	3 4 1 1	3 3 1 1	
2.0	3 3 1 2	3 3 1 1	3 4 1 1	2 2 1 1	

Kinetin

(b). Lamina

6-BAP \ IAA	0.0	1.0	2.0	5.0	Control
1.0	2 2 1 1	2 3 1 1	2 3 1 2	3 4 1 3	1 2 1 1
2.0	3 2 1 2	2 3 1 1	1 2 1 1	2 1 1 1	
1.0	3 2 1 1	2 2 1 2	2 2 1 1	2 2 1 1	
2.0	2 2 1 1	1 2 1 1	1 3 1 1	1 2 1 1	

Kinetin

Scale

G	C
S	R

G=Growth C=Colour

S=Shoots R=Roots

Table 22

Comparative responses of (a). stem and (b). lamina derived callus to media containing different growth regulators (mg l^{-1}) at 28 days.

(a). Stem

6-BAP \ IAA	0.0	1.0	2.0	5.0	Control
1.0	3 3 1 1	3 2 1 1	3 3 1 1	3 3 1 1	1 1 1 1
2.0	2 2 1 1	3 4 1 1	3 4 1 1	3 4 1 1	
Kinetin 1.0	2 2 1 1	3 4 1 1	4 3 1 1	3 3 1 1	
2.0	3 3 1 1	3 2 1 2	3 4 1 1	3 2 1 1	

(b). Lamina

6-BAP \ IAA	0.0	1.0	2.0	5.0	Control
1.0	1 2 1 1	2 1 1 1	2 2 1 2	3 3 1 2	1 2 1 1
2.0	2 3 1 2	3 2 1 1	1 1 1 1	2 1 1 1	
Kinetin 1.0	2 2 1 1	2 1 1 2	2 2 1 1	2 2 1 1	
2.0	2 2 1 1	1 2 1 1	1 2 1 1	1 3 1 1	

Scale

G	C
S	R

G=Growth C=Colour

S=Shoots R=Roots

Table 23

Comparative responses of hypocotyl derived callus to media containing different growth regulators (mg l^{-1}) at 14 days.

6-BAP

IAA	0.0	1.0	2.0	5.0	8.0	10.0	12.0
0.0	1 1 1 1	2 3 1 3	2 2 1 1	1 1 3 2	2 2 1 1	1 2 1 2	1 2 1 2
1.0	2 3 1 1	3 2 1 1	3 2 1 1	2 3 1 1	2 2 1 1	2 2 1 1	3 3 1 1
5.0	2 2 1 1	2 3 1 1	2 2 1 2	2 3 1 1	2 1 1 1	2 2 1 1	2 2 1 1
10.0	3 3 1 3	3 3 1 1	3 3 1 1	3 3 1 1	3 3 1 1	3 4 1 1	2 4 1 1

Zeatin

IAA	0.0	1.0	2.0	5.0	8.0	10.0	12.0
0.0	1 1 1 1	2 2 1 1	2 2 2 1	2 2 1 1	2 3 3 2	1 2 2 1	1 2 2 2
1.0	2 2 1 1	2 3 1 1	3 3 2 2	3 2 2 2	2 2 1 1	3 3 2 2	3 2 1 1
5.0	2 2 2 2	2 3 1 1	2 2 1 2	2 2 1 2	2 3 1 2	2 2 2 2	2 2 1 1
10.0	2 3 1 3	3 3 2 2	3 3 1 1	3 2 1 1	3 1 1 1	1 1 1 1	2 3 1 1

Scale

G	C
S	R

G=Growth C=Colour

S=Shoots R=Roots

Table 24

Comparative responses of hypocotyl derived callus to media containing different growth regulators (mg l^{-1}) at 21 days.

6-BAP

IAA \ 6-BAP	0.0	1.0	2.0	5.0	8.0	10.0	12.0
0.0	2 2 1 1	3 3 1 1	2 2 5 2	2 2 5 2	2 2 1 2	1 2 2 2	1 2 2 1
1.0	2 3 1 1	3 2 1 1	3 2 1 1	3 3 2 1	2 3 4 1	3 3 2 1	3 2 1 1
5.0	3 2 1 1	3 3 1 1	3 2 2 2	3 3 1 2	3 2 1 2	2 2 2 2	2 2 1 2
10.0	4 3 1 3	3 3 1 1	3 2 3 1	3 3 2 3	3 2 3 1	3 4 2 2	3 2 1 1

Zeatin

IAA \ Zeatin	0.0	1.0	2.0	5.0	8.0	10.0	12.0
0.0	2 3 1 1	2 2 1 1	2 3 2 2	2 3 4 1	2 2 3 2	1 2 3 1	1 2 2 2
1.0	2 2 1 1	2 3 1 3	3 3 2 3	3 2 2 3	2 2 2 2	3 3 2 2	3 2 1 1
5.0	2 2 2 3	3 3 3 1	3 2 3 2	3 2 3 2	3 3 1 1	2 2 1 2	2 2 1 1
10.0	2 3 1 3	3 3 2 3	3 3 3 3	3 3 2 2	3 3 4 2	3 4 1 1	2 4 1 1

Scale

G	C
S	R

G=Growth C=Colour

S=Shoots R=Roots

Table 25

Comparative responses of hypocotyl derived callus to media containing different growth regulators (mg l^{-1}) at 28 days.

6-BAP

IAA \ 6-BAP	0.0	1.0	2.0	5.0	8.0	10.0	12.0
0.0	1 2 1 1	1 1 1 1	2 2 5 2	1 2 5 2	1 2 5 2	1 2 2 3	1 2 2 1
1.0	2 3 1 1	3 2 1 1	3 2 1 1	3 3 2 1	1 3 4 1	3 3 2 1	3 2 1 1
5.0	2 2 1 1	3 3 1 2	3 3 2 2	3 3 2 2	2 3 1 2	2 2 2 2	2 3 1 3
10.0	3 4 1 3	3 3 2 1	3 3 3 1	3 3 2 3	3 2 3 1	3 2 2 2	3 2 1 1

Zeatin

IAA \ Zeatin	0.0	1.0	2.0	5.0	8.0	10.0	12.0
0.0	2 3 1 1	2 2 1 1	1 3 5 1	1 3 4 2	1 3 4 2	2 2 4 1	1 2 3 3
1.0	1 1 1 1	2 3 1 3	2 3 2 3	2 2 3 3	2 2 2 2	2 3 2 2	3 2 1 1
5.0	2 2 2 3	2 3 3 1	3 2 3 2	3 2 3 2	3 3 3 2	2 2 1 2	2 2 3 1
10.0	3 3 1 3	3 2 3 3	3 4 3 2	2 4 2 2	3 3 4 2	3 4 2 2	2 4 2 2

Scale

G	C
S	R

G=Growth C=Colour

S=Shoots R=Roots

REFERENCES

AGRAWAL, S., and CHANDRA, N. (1983). Differentiation of multiple shoot buds and plantlets in cultured embryos of *Capsicum annuum* L. var. Mathania. Current Science, **52**, 645–646.

AITCHISON, P.A., McCLEOD, A.J., and YEOMAN, M.M. (1977). Growth patterns in tissue (callus) cultures. In Plant Tissue and Cell Culture, ed. H.E. Street, pp. 267–306. Botanical Monographs, Vol.11, Blackwell Scientific Publications, Oxford.

ALVEY, N., GALWAY, N., and LANE, P. (1982). An Introduction to Genstat. Academic Press, London.

ARNISON, G., and BOLL, W.G. (1975). Isoenzymes in cell cultures of bush bean (*Phaseolus vulgaris* cv. Contender): Isoenzymatic differences between stock suspension cultures derived from a single seedling. Canadian Journal of Botany, **53**, 261–271.

BAYLISS, M.W. (1980). Chromosomal variation in plant tissues in culture. International Review of Cytology Supplement, **11A**, 113–144.

BERGUONIOUX, C., PERENNES, C., BROWN, S.C., and GADAL, P. (1988). Cytometric analysis of growth-regulator-dependent transcription and cell cycle progression in *Petunia* protoplast cultures. Planta, **175**, 500–508.

BERLIN, J., KUKOSCHKE, K.G., and KNOBLOCH, K.-H. (1981). Selection of tobacco cell lines with high levels of cinnamoyl putrescines. Planta Medica, **42**, 173–180.

BEVAN, M., and NORTHCOTE, D.H. (1981). Some rapid effects of synthetic auxins on mRNA levels in cultured plant cells. Planta, **152**, 32–35.

BLAKELY, L.W., and STEWARD, F.C. (1964). Growth and organised development of cultured cells. VII. Cellular variation. American Journal of Botany, **51**, 809–820.

BRADFORD, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye-binding. Analytical Biochemistry, **72**, 248–254.

BROWN, S. (1984). Analysis and sorting of plant material by flow cytometry. Physiol. Veg., **22**, 341–349.

CARCELLER, M., DAVEY, M.R., FOWLER, M.W., and STREET, H.E. (1971). The influence of sucrose, 2,4-D and kinetin on the growth, fine structure and lignin content of cultured sycamore cells. Protoplasma, **73**, 367-385.

CHAPRIN, N., and ELLIS, B.E. (1984). Microspectrophotometric evaluation of rosmarinic acid accumulation in single cultured plant cells. Canadian Journal of Botany, **62**, 2278-2282.

CHATURVEDI, H.C., and MITRA, G.C. (1975). A shift in the morphogenetic pattern in *Citrus* callus tissue during prolonged culture. Molecular and General Genetics, **181**, 254-258.

CHOWDHURY, M.K.U., SHAEFFER, G.W., SMITH, R.L., and MATTHEWS, B.F. (1988). Molecular analysis of organelle DNA of different subspecies of rice and the genomic stability of mitochondrial DNA in tissue cultured cells of rice. Theoretical Applied Genetics, **76**, 533-539.

COLLIN, H.A. (1987). Determinants of yield of secondary products in plant tissue cultures. In Advances in Botanical Research, ed. J. Callow, pp. 145-187. Academic Press, New York.

CONSTABEL, F., SHYLUK, J.P., and GAMBORG, O.L. (1971). The effect of hormones on anthocyanin accumulation in cell cultures of *Haplopappus gracilis*. Planta, **96**, 306-316.

CONSTABEL, F., RAMBOLD, S., CHATSON, K.B., KURZ, W.G.M., and KUTNEY, J.P. (1981). Alkaloid production in *Catharanthus roseus* (L.) G.Don. VI. Variation in alkaloid spectra of cell lines derived from one single leaf. Plant Cell Reports, **1**, 3-5.

CRECHE, J., GUILLER, J., ANDREU, F., GRAS, M., CHENIEUX, J.-C., and RIDEAU, M. (1987). Variability in tissue cultures of *Choisya ternata* originating from a single tree. Phytochemistry, **26**, 1947-1953.

CURTIN, B. (1983). Harvesting profitable products from plant tissue culture. Bio/Technology, **1**, 649-657.

DALTON, C.C, and PEEL, E. (1983). Product formation and plant cell specialization: A case study of photosynthetic development in plant cell cultures. Progress in Industrial Microbiology, **17**, 109-166.

DAVEY, M.R., FOWLER, M.W., and STREET, H.E. (1971). Cell clones contrasted in growth, morphology and pigmentation isolated from a callus culture of *Atropa belladonna* var. *lutea*. Phytochemistry, **10**, 2559-2575.

DAVIES, M.E. (1972). Polyphenol synthesis in cell suspension cultures of Paul's Scarlet Rose. Planta, **104**, 50-65.

DENNIS, E.S., BRETTELL, R.I.S., and PEACOCK, W.J. (1987). A tissue culture induced Adh1 cell mutant of maize results from a single base change. Molecular and General Genetics, **210**, 181-183.

DEUS-NEUMANN, B., and ZENK, M.H. (1984). Instability of indole alkaloid production in *Catharanthus roseus* cell suspension cultures. Planta Medica, **50**, 427-431.

DHOOT, G.K., and HENSHAW, G.G. (1977). Organisation and alkaloid production in tissue cultures of *Hyocyamus niger*. Annals of Botany, **41**, 943-949.

DIX, P.H. (1986). Cell line selection. In Plant Cell Culture Technology, ed. M.M. Yeoman, pp. 143-201. Botanical Monographs, Vol. 23. Blackwell Scientific Publications, Oxford.

DOUGALL, D.K. (1980). Nutrition and Metabolism. In Plant Tissue Culture as a Source of Biochemicals, ed. E.J. Staba, pp. 21-58. CRC Press, Boca Raton, Florida.

DOUGALL, D.K. (1981). Tissue culture and the study of secondary natural products. In The Biochemistry of Plants: Secondary Plant Products, Vol. 7, ed. E.E. Conn, pp. 21-34. Academic Press, New York.

DOUGALL, D.K. (1985). Chemicals from plant cell cultures: Yields and variation. In Biotechnology in Plant Science, eds. M. Zaitlin, P. Day and A. Hollandear, pp. 179-190. Academic Press, New York.

DOUGALL, D.K. (1987). Cell cloning and the selection of high yielding strains. In Cell Culture and Somatic Cell Genetics of Plants, Vol. 4, eds. F. Constabel and I.K. Vasil, pp. 117-124. Academic Press, New York.

DOUGALL, D.K., and WEYRAUCH, K.W. (1980). Growth and anthocyanin production by carrot suspension cultures grown under chemostat conditions with phosphate as the limiting nutrient. Biotechnology and Bioengineering, **25**, 581-594.

DOUGALL, D.K., JOHNSON, J.M., and WHITTEN, G.H. (1980). A clonal analysis of anthocyanin accumulation by cell cultures of wild carrot. Planta, **149**, 292-297.

ELLIS, B.E. (1985). Characterisation of clonal cultures of *Anchusa Officinalis* derived from single cells of known productivity. Journal of Plant Physiology, **119**, 149-158.

EVANS, D.A., and REED, S.M. (1981). Cytogenetic techniques. In Plant Tissue Culture: Methods and Applications in Agriculture, ed. T.A. Thorpe, pp. 213-240 Academic Press, New York.

EVANS, D.A., SHARP, W.R., and FLICK, C.E. (1981). Plant regeneration from cell cultures. Horticultural Review, **2**, 214-314.

EVANS, D.A., SHARP, W.R., and MEDINA-FILHO, H.P. (1984). Somaclonal and gametoclonal variation. American Journal of Botany, **71**, 759-744.

FLICK, C.E., EVANS, D.A., and SHARP, W.R. (1983). Organogenesis. In Handbook of Plant Cell Culture, Vol. 1, Techniques for propagation and breeding, eds. D.A. Evans, W.R. Sharp, P.V. Ammirato, and Y. Yamada, pp. 13-81. MacMillan Publishing Co., New York, London.

FOWLER, M.W. (1983). Commercial applications and economic aspects of mass plant cell culture. In Plant Biotechnology, eds. S.H. Mantell and H. Smith, pp. 3-37. Cambridge University Press, Cambridge.

FURUYA, T., KOJIMA, H., and SYONO, K. (1971). Regulation of nicotene biosynthesis by auxins in tobacco callus cultures. Phytochemistry, **10**, 1529-1532.

GOULD, A.R. (1986). Factors controlling generation of variability *in vitro*. In Cell Culture and Somatic Cell Genetics of Plants, Vol. 3, Plant Regeneration and Genetic Variability, ed. I.K. Vasil, pp. 549-567. Academic Press, London and New York.

GOULD, A.R., EVERETT, N.P., WANG, T.L., and STREET, H.E. (1981). Studies on the control of the cell cycle. I. Effects of nutrient limitation and nutrient starvation. Protoplasma, **106**, 1–13.

GUNAY, A.L., and RAO, P.S. (1978). *In vitro* plant regeneration from hypocotyl and cotyledon explants of red pepper (*Capsicum*). Plant Science Letters, **11**, 365–372.

HADDON, L.E., and NORTHCOTE, D.H. (1976). Quantitative measurement of the course of bean callus differentiation. Journal of Cell Science, **17**, 11–19.

HADDON, L.E., and NORTHCOTE, D.H. (1975). The effect of growth conditions and origin of tissue on the ploidy and morphogenetic potential of tissue cultures of bean. Journal of Experimental Botany, **27**, 1031–1051.

HALL, R.D., and YEOMAN, M.M. (1986). Temporal and spatial heterogeneity in the accumulation of anthocyanins in cell cultures of *Catharantus roseus* (L.) G.Don. Journal of Experimental Botany, **37**, 48–60.

HALL, R.D., and YEOMAN, M.M. (1987). Intercellular and intercultural heterogeneity in secondary metabolite accumulation in cultures of *Catharanthus roseus* following cell line selection. Journal of Experimental Botany, **38**, 1391–1398.

HALL, R.D., HOLDEN, M.A., and YEOMAN, M.M. (1987). The accumulation of phenylpropanoid and capsaicinoid compounds in cell cultures and whole fruit of the chilli pepper, *Capsicum frutescens* Mill. Plant Cell, Tissue and Organ Culture, **8**, 163–176.

HARBORNE, J.B. (1976). Phytochemical Methods: A Guide to Modern Techniques in Plant Analysis. Chapman and Hall, London.

HEISER, C.B. (1976). Peppers. In Evolution of Crop Plants, ed. N.W. Simmonds, pp. 265–268. Longman, London and New York.

HOLDEN, M.A., HALL, R.D., LINDSEY, K., and YEOMAN, M.M. (1987). Capsaicin biosynthesis in cell cultures of *Capsicum frutescens*. In Plant and Animal Cells, Process Possibilities, eds. C. Webb and F. Mavituna, pp. 45–63. Ellis Horwood Books in Biochemistry and Biotechnology, Ellis Horwood Limited, Chichester.

JAHNEN, W., and HAHLBROCK, K. (1988a). Cellular localisation of nonhost resistance reactions of parsley (*Petroselinum crispum*) to fungal infection. Planta, **173**, 197–204.

JAHNEN, W., and HAHLBROCK, K. (1988b). Differential regulation and tissue specific distribution of enzymes of phenylpropanoid pathways in developing parsley seedlings. Planta, **173**, 453–458.

JONES, D.H. (1984). Phenylalanine ammonia-lyase: regulation of its induction and role in plant development. Phytochemistry, **23**, 1349–1359.

KADKADE, P.G. (1982). Growth and podophyllotoxin production in callus tissues of *Podophyllum peltatum*. Plant Science Letters, **25**, 107–115.

KARP, A., and MADDOCK, S.E. (1984). Chromosome variation in wheat plants regenerated from cultured immature embryos. Theoretical and Applied Genetics, **63**, 265–272.

KARP, A., and BRIGHT, S.W.J. (1985). On the causes and origins of somaclonal variation. Oxford Survey Plant Molecular Cell Biology, **2**, 199–234.

KETEL, D.H., BRETELER, H., and GROOT, B. (1985). Effect of explant origin on growth and differentiation of calli from *Tagetes* species. Journal of Plant Physiology, **118**, 327–333.

KING, P.J. (1980). Cell proliferation and growth in suspension cultures. International Review of Cytology Supplement, **11A**, 25–53.

KINNERSLEY, A.M., and DOUGALL, D.K. (1980). Correlation between nicotine content of tobacco plants and callus cultures. Planta, **149**, 205–206.

KNOBLOCH, K.H., and BERLIN, J. (1980). Influence of medium composition on the formation of secondary compounds in cell suspension cultures of *Catharanthus roseus*. Zeitschrift Naturforsch, **35c**, 551–556.

KNOBLOCH, K.H., and BERLIN, J. (1981). Phosphate-mediated regulation of cinnamoyl biosynthesis in cell suspension cultures of *Nicotiana tabacum*. Planta Medica, **42**, 167–172.

KNOBLOCH, K.H., and BERLIN, J. (1983). Influence of phosphate on the formation of the indole alkaloids and phenolic compounds in cell suspension cultures of *Catharanthus roseus*. Plant Cell, Tissue and Organ Culture, **2**, 333-340.

KNOBLOCH, K.H., BAST, G., and BERLIN, J. (1982). Medium- and light-induced formation of serpentine and anthocyanins in cell suspension cultures of *Catharanthus roseus*. Phytochemistry, **21**, 591-594.

KRUTH, H.S. (1982). Flow cytometry:rapid biochemical analysis of single cells. Analytical Biochemistry, **125**, 225-242.

LARKIN, P.J., and SCOWCROFT, W.R. (1981). Somaclonal variation-a novel source of variabilty from cell culture for plant improvement. Theoretical and Applied Genetics, **60**, 197-214.

LARKIN, P.J., BRETTELL, R.I.S, RYAN, S.A., DAVIES, P.A., PALLOTTA, M.A., and SCOWCROFT, W.R. (1985). Somaclonal variation: impact on plant biology and breeding strategies. In Biotechnology in Plant Science, eds. M. Zaitlin, P. Day and A. Hollaender, pp. 83-100. Academic Press, New York.

LEE, M., and PHILLIPS, R.L. (1988). The chromosomal basis of somaclonal variation. Annual Review of Plant Physiology and Molecular Biology, **39**, 413-437.

LINDSEY, K. (1982). Studies on the growth and metabolism of plant cells cultured on fixed-bed reactors. PhD. Thesis, University of Edinburgh, Edinburgh.

LINDSEY, K. (1985). Manipulation, by nutrient limitation, of the biosynthetic activity of immobilized cells of *Capsicum frutescens* Mill. cv. annuum. Planta, **165**, 126-133.

LINDSEY, K. (1986). Incorporation of ^{14}C phenylalanine and ^{14}C cinnamic acid into capsaicin in cultured cells of *Capsicum frutescens*. Phytochemistry, **25**, 2793-2801.

LINDSEY, K., and YEOMAN, M.M. (1984a). The synthetic potential of immobilized cells of *Capsicum frutescens* Mill. cv. annuum. Planta, **162**, 495-501.

LINDSEY, K., and YEOMAN, M.M. (1984b). The viability and biosynthetic activity of cells of *Capsicum frutescens* Mill. cv. annuum immobilized in reticulate polyurethane. Journal of Experimental Botany, **35**, 1684–1696.

LINDSEY, K., and YEOMAN, M.M. (1985). Dynamic aspects of plant cell culture. In Cell Culture and Somatic Cell Genetics of Plants: A Comprehensive Treatise, ed. I.K. Vasil, pp. 61–101. Academic Press, London and New York.

LINDSEY, K., YEOMAN, M.M., BLACK, G.M., and MAVITUNA, F. (1983). A novel method for the immobilization and culture of plant cells. FEBS letters, **155**, 143–149.

LORZ, H., and SCOWCROFT, W.R. (1983). Variability among plants and their progeny regenerated from protoplasts of Su/Su heterozygotes of *Nicotiana tabacum*. Theoretical and Applied Genetics, **66**, 67–75.

MANTELL, S.H., and SMITH, H. (1983). Cultural factors that influence secondary metabolite accumulations in plant cell and tissue cultures. In Plant Biotechnology, eds. S.H. Mantell and H. Smith, pp. 75–110. Cambridge University Press, Cambridge.

MARTIN, S.M. (1980). Environmental factors: B. Temperature, aeration and pH. In Plant Tissue Culture as a Source of Biochemicals, ed. E.J. Staba, pp. 143–148. Boca Raton, Florida: CRC Press.

MAVITUNA, F., PARK, J.M., WILKINSON, A.K., and WILLIAMS, P.D. (1987). Characteristics of immobilized plant-cell reactors. In Plant and Animal Cells, Process Possibilities, eds. C. Webb and F. Mavituna, pp. 92–115. Ellis Horwood Limited, Chichester.

McCOY, T.J., PHILLIPS, R.L., and RINES, H.W. (1982). Cytogenetic analysis of plants regenerated from oat (*Avena sativa*) tissue cultures: high frequency of partial chromosome loss. Canadian Journal of Genetics and Cytology, **24**, 37–50.

MEINS, F. (1983). Heritable variation in plant cell culture. Annual Review of Plant Physiology, **34**, 327–346.

MEINS, F. (1986). Determination and morphogenetic competence in plant tissue culture. In Plant Cell Culture Technology, ed. M.M. Yeoman, pp. 7–25. Botanical Monographs, Vol. 23. Blackwell Scientific Publications, Oxford.

MEINS, F., and BINNS, A.N. (1977). Epigenetic variation of cultured somatic cells: Evidence for gradual changes in the requirement for factors promoting cell division. Proceedings of the National Academy of Sciences, U.S.A, **74**, 2928–2932.

MEINS, F., LUTZ, J., and FOSTER, R. (1980). Factors influencing the incidence of habituation for cytokinin of tobacco pith tissue in culture. Planta, **150**, 264–268.

MIKAMI, T., and KINOSHITA, T. (1988). Genotypic effects on the callus formation from different explants of rice, *Oryza sativa* L., Plant Cell, Tissue and Organ Culture, **12**, 311–314.

MOK, M.C., and MOK, D.W.S. (1977). Genotypic responses to auxins in tissue cultures of *Phaseolus*. Physiologia Plantarum, **40**, 261–264.

MOK, M.C., GABELMAN, W.H., and SKOOG, F. (1976). Carotenoid synthesis in tissue cultures of *Daucus carota* L. Journal of the American Society of Horticultural Science, **101**, 442–449.

MORRISON, R.A., KONING, R.E., and EVANS, D.A. (1986). Anther culture of an interspecific hybrid of *Capsicum*. Journal of Plant Physiology, **126**, 1–9.

NAGEL, M., and REINHARD, E. (1975). Das Aetherische Oel der Calluskulturen von *Ruta graveolens* L. Die Zuzammensetzung des Oeles. Planta Medica, **27**, 151–158.

NETTLESHIP, L., and SLAYTOR, M. (1974). Adaptation of *Peganum harmala* callus to alkaloid production. Journal of Experimental Botany, **25**, 1114–1123.

NISHIMAKI, T., and NOZUE, M. (1985). Isolation and culture of protoplasts from high anthocyanin-producing callus of sweet potato. Plant Cell Reports, **4**, 248–251.

OGINO, T., HIROAKA, N., and TABATA, M. (1978). Selection of high nicotine-producing cell lies of tobacco by single cell cloning. Phytochemistry, **17**, 1907–1910.

ORTON, T.J. (1980). Chromosomal variability in tissue cultures of regenerated plants of *Hordeum*. Theoretical and Applied Genetics, **64**, 223–230.

ORTON, T.J. (1983). Experimental approaches to the study of somaclonal variation. Plant Molecular Biology Reports, **1**, 67-76.

OZEKI, Y., and KOMAMINE, A. (1985). Changes in activities of enzymes involved in general phenylpropanoid metabolism during the induction and reduction of anthocyanin synthesis in a carrot suspension culture as regulated by 2,4-D. Plant Cell Physiology, **26**, 903-911.

PARKER, R.E. (1973). Introductory statistics for biology. Studies in Biology No. 43, Edward Arnold Publishers, London.

PESCHKE, V.M., PHILLIPS, R.L., and GENGENBACH, B.G. (1987). Discovery of transposable element activity among progeny of tissue culture derived maize plants. Science, **238**, 804-807.

PETIARD, V., BAUBAULT, C., BARIAUD, A., HUTIN, M., and COURTOIS, D. (1985). Studies on variability in plant tissue cultures for alkaloid production in *Catharanthus roseus* and *Papaver somniferum* callus cultures. In Primary and Secondary Metabolism in Plant Cell Cultures, eds. K.-H. Neumann, W. Barz and E. Reinhard, pp. 133-142. Springer-Verlag, Berlin and New York.

PHILLIPS, R., and HENSHAW, G.G. (1977). The regulation and synthesis of phenolics in stationary phase cell cultures of *Acer pseudoplatanus* L. Journal of Experimental Botany, **42**, 245-256.

PICKERSGILL, B. (1987). Genetic resources of *Capsicum*-a global plan of action. IPBGR Report, IBPGR secretariat, Rome.

PIJNACKEN, L.P., SCREE RAMULU, K., DIJKHUIS, P., and FERWERDA, M.A. (1989). Flow cytometric and karyological analysis of polysomaty and polyploidization during callus formation from leaf segments of various potato genotypes. Theoretical and Applied Genetics, **77**, 102-110.

POTRYKUS, I., PASZKOWSKI, J., and SAUL, M. (1987). Direct gene transfer to plants: facts and future. In Plant Tissue and Cell Culture, eds. C.E. Green, D.A. Somers, W.P. Hackett and D.D. Biesboer, pp. 289-302. Alan. R. Liss, Inc., New York.

PURSEGLOVE, J.W. (1968). Tropical Crops : Dicotyledons. Longman, London.

RAMULU, K.S., and DIJKHUIS, P. (1986). Flow cytometric analysis of polysomaty and *in vitro* genetic instability in potato. Plant Cell Reports, **3**, 234–237.

RYAN, S.A., LARKIN, P.J., and ELLISON, P. (1987). Somaclonal variation in some agronomic and quality characters in wheat. Theoretical and Applied Genetics, **74**, 77–82.

SATO, F., and YAMADA, Y. (1984). High berberine producing cultures of *Coptis japonica* cells. Phytochemistry, **23**, 281–285.

SCOWCROFT, W.R. (1984). Genetic variability in tissue culture: Impact on germplasm conservation and utilisation. IBPGR report, IPBGR Secretariat, Rome.

SCOWCROFT, W.R., DAVIES, P., RYAN, S.A., BRETTELL, R.I.S., PALLOTTA, M.A., and LARKIN, P.J. (1985). The analysis of somaclonal mutants. In Plant Genetics, ed. M. Freeling, pp. 799–815. Alan. R. Liss, New York.

SELBY, C., and COLLIN, H.A. (1976). Clonal variation in growth and flavour production in tissue cultures of *Allium cepa* L. Annals of Botany, **40**, 911–918.

SCHENK, R.U., and HILDEBRANDT, A.C. (1972). Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Canadian Journal of Botany, **50**, 199–204.

SCHWEIGER, H.-G., DIRK, J., KOOP, H.-U., KRANZ, E., NEUHAUS, G., SPANGENBERG, G., and WOLFF, D. (1987). Individual selection, culture and manipulation of higher plant cells. Theoretical and Applied Genetics, **73**, 769–783.

SHIRZADEGAN, M., CHRISTEY, M., EARLE, E.D., and PALMER, J.D. (1989). Rearrangement, amplification, and assortment of mitochondrial DNA molecules in cultured cells of *Brassica campestris*. Theoretical and Applied Genetics, **77**, 17–25.

SIEVERT, R.C., and HILDEBRANDT, A.C. (1965). Variations within single cell clones of tobacco tissue cultures. Annals of Botany, **52**, 742–750.

SIGMA CHEMICAL COMPANY LIMITED, Fancy Road, Poole, Dorset, England.

SINGH, B.D., HARVEY, B.L., KAO, K.N., and MILLER, R.A. (1975). Karyotypic changes and selection pressure in *Haplopappus gracilis* suspension cultures. Canadian Journal of Genetics and Cytology, **17**, 109–116.

SKIRVIN, R.M. (1978). Natural and induced variation in tissue culture. Euphytica, **27**, 241-266.

SNEDECOR, G., and COCHRAN, W. (1967). Statistical Methods. Iowa State University Press, Iowa.

SNIJMAN, D.A., NOEL, A.R.A., BORNMAN, C.H., and ABBOTT, J.G. (1977). *Nicotiana tabacum* callus studies. II. Variability in cultures. Z.Pflanzenphysiol, **82**, 367-370.

SPEAKE, T., McCLOSKEY, P., SMITH, W., SCOTT, T., and HUSSEY, H. (1964). Isolation of nicotine from cell cultures of *Nicotiana tabacum*. Nature, **201**, 614-615.

STABA, E.J. (1980). Plant Tissue Culture as a Source of Biochemicals. CRC Press, Boca Raton, Florida.

STREET, H.E. (1977). Differentiation in cell and tissue cultures- regulation at the molecular level. In Regulation and Developmental Processes in Plants, eds. H.R. Schutte and D. Gross, pp. 192-218. VEB Gustav Fischer Verlag, Jena.

SUNDERLAND, N. (1977). Nuclear cytology. In Plant Tissue and Cell Culture, ed. H.E. Street, pp. 177-205. Botanical Monographs, Vol. 11, Blackwell Scientific Publications, Oxford.

TABATA, M., and HIRAOKA, N. (1976). Variation in alkaloid production in *Nicotiana rustica* callus cultures. Physiologia Plantarum, **38**, 19-23.

TABATA, M., and FUJITA, Y. (1985). Production of shikonin by plant cell cultures. In Biotechnology in Plant Science : Relevance to Agriculture in the Eighties, eds. M. Zaitlin, P. Day and A. Hollaender, pp. 207-218. Academic Press, New York.

TABATA, M., YAMAMOTO, H., HIRAOKA, N., and KONOSHIMA, M. (1972). Organisation and alkaloid production in tissue cultures of *Scopolia parviflora*. Phytochemistry, **11**, 949-955.

TALBOT, H. (1981). An introductory Guide to Genstat. ERCC, Edinburgh University.

TANAKA, Y., KOJIMA, M., and URITANI, I. (1974). Properties, development and cellular localisation of cinnamic acid 4-hydroxylase in cut-injured sweet potato. Plant and Cell Physiology, **15**, 843-854.

TORREY, J.G. (1965). Cytological evidence of cell selection by plant tissue. In Plant Tissue Culture, eds. P.R. White and A.R. Grove, pp. 473-484. McCutchan, Berkeley, California.

TREMOUILLAUX-GUILLER, J.T., ANDREU, F., CRECHE, J., CHENIEUX, J.-C., and RIDEAU, M. (1987). Variability in tissue cultures of *Choisya ternata*. II. Alkaloid accumulation in protoclones and aggregate clones obtained from established strains. Plant Cell Reports, **6**, 375-378.

TREMOUILLAUX-GUILLER, J.T., KODJA, H., ANDREU, F., CRECHE, J., CHENIEUX, J.-C., and RIDEAU, M. (1988). Variability in tissue culture of *Choisya ternata*. III. Comparing alkaloid production in cell lines obtained by various strategies. Plant Cell Reports, **7**, 456-458.

VASIL, I.K. (1986). Cell Culture and Somatic Cell Genetics of Plants: Plant Regeneration and Genetic Variability. Academic Press, London and New York.

WERNICKE, W., and BRETTELL, R. (1980). Somatic embryogenesis from *Sorghum bicolor* leaves. Nature, **287**, 138-139.

WESTCOTT, R.J., and HENSHAW, G.G. (1976). Phenolic synthesis and PAL activity in suspension cultures of *Acer pseudoplatanus* L. Planta, **131**, 67-73.

WITHERS, L.A. (1986). Cryopreservation and genebanks. In Plant Cell Culture Technology, ed. M.M. Yeoman, pp. 96-140. Botanical Monographs, Vol. 23. Blackwell Scientific Publications, Oxford.

YEOMAN, M.M. (1987). Bypassing the plant. Annals of Botany, **60**, 157-174.

YEOMAN, M.M., and DAVIDSON, A.W. (1971). Effect of light on cell division in developing callus cultures. Annals of Botany, **35**, 1085-1100.

YEOMAN, M.M., and FORCHE, E. (1980). Cell proliferation and growth in callus cultures. International Review of Cytology Supplement, **11A**, 1-24.

YEOMAN, M.M., MIEDZYBRODZKA, M.B., LINDSEY, K., and McLAUCHLAN, W.R. (1980). The synthetic potential of cultured plant cells. In Plant Cell Cultures: Results and Perspectives, eds. F. Sala, B. Parisi, R. Cella and O. Cifferi, pp. 327-343. Elsevier North Holland, Amsterdam.

YEOMAN, M.M., LINDSEY, K., and HALL, R.D. (1982a). Differentiation as a prerequisite for the production of secondary metabolites. In Proceedings of the Plant Cell Culture Conference, pp. 1-7. Oyez Scientific and Technical Services, London.

YEOMAN, M.M., LINDSEY, K., MIEDZYBRODZKA, M.B., and McLAUCHLAN, W.R. (1982b). Accumulation of secondary products as a facet of differentiation in plant cell and tissue cultures. In Differentiation *in vitro*, eds. M.M. Yeoman and D.E.S Truman. British Society for Cell Biology Symposium, Vol. 4, pp. 65-82.

ZENK, M.H., EL-SHAGI, H., and SCHULTE, U. (1975). Anthraquinone production in cell suspension cultures of *Morinda citrofolia*. Planta Medica Supplement, 79-101.

ZENK, M.H., El-SHAGI, H., ARENS, H., STOCKIGT, J., WEILER, E.W., and DEUS, B. (1977). Formation of the indole alkaloids serpentine and ajmalicine in cell suspension cultures of *Catharanthus roseus*. In Plant Tissue Culture and its Biotechnological Application, eds. W. Barz, E. Reinhard and M.H. Zenk, pp. 27-43. Springer-Verlag, Berlin and New York.

ZUCKER, M. (1965). Induction of phenylalanine deaminase by light and its relation to chlorogenic acid synthesis in potato tuber tissue. Plant Physiology, **40**, 779-784.

PUBLICATIONS

HOLDEN, P.R., AITKEN, M., LINDSEY, K., and YEOMAN, M.M. (1986). Variability and stability of cell cultures of *Capsicum frutescens*. In Secondary Metabolism in Plant Cell Cultures, eds. P. Morris, A.H. Scragg, A. Stafford, and M.W. Fowler, pp. 237–243. Cambridge University Press, Cambridge.

HOLDEN, P.R., HOLDEN, M.A., and YEOMAN, M.M. (1988). Variation in the secondary metabolism of cultured plant cells. In Manipulating Secondary Metabolism in Culture, eds. R.J. Robins and M.J.C. Rhodes, pp. 15–29. Cambridge University Press, Cambridge.

HOLDEN, P.R., HOLDEN, M.A., and YEOMAN, M.M. (1988). The effects of fungal elicitation on secondary metabolism in cell cultures of *Capsicum frutescens*. In Manipulating Secondary Metabolism in Culture, eds. R.J. Robins and M.J.C. Rhodes, pp. 67–72. Cambridge University Press, Cambridge.

NEEDS TO BE STUCK IN

31. VARIABILITY AND STABILITY OF CELL CULTURES OF CAPSICUM FRUTESCENS

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INTRODUCTION

Temporal and spatial variation in morphology and metabolism within plant cell cultures creates difficulties when trying to maintain cells in vitro for experimental purposes. Progress in the development and exploitation of many advanced in vitro techniques is often prevented by the inability to overcome problems arising in apparently basic procedures such as maintenance of growth or regeneration of plants from manipulated cells. It is a well known fact that with increasing time in culture cells exhibit gross changes in their karyotype as well as a reduction or loss of their biosynthetic or regenerative potentials (Skirvin, 1978; Thomas et al, 1979).

Direct genetic changes are very common in cultured plant cells and there are many well documented examples of increased ploidy levels (Bennici et al, 1968; Renfroe & Berlyn, 1985) as well as structural rearrangements of chromosomes (Mitra & Stewart, 1961; Bayliss, 1980). Likewise the incidence of epigenetic change is very high although not so readily recognised as chromosomal modifications (Meins & Binns, 1977; Meins, 1983). The reduction or loss in the capacity of the cells to produce secondary metabolites while in culture is also widely reported (Tabata & Hiraoka, 1976; Deus-Neumann & Zenk, 1984). This instability has prevented progress in the commercial production of useful compounds by mass cell culture (Scragg & Fowler, 1985). Similarly the gradual loss in regenerative potential with time in tissue culture has caused problems with genetic manipulation (Thomas et al., 1979; Dale, 1983) and germplasm storage (Withers, 1983).

The variation expressed in plant cell cultures, though considered to be detrimental to the stability and homogeneity of the cultures, can be exploited as a source of genetic diversity. If cultured cells can be induced to differentiate back to the whole plant then variation which has arisen in culture can be manifested in the regenerated plant. This phenomenon, termed somaclonal variation (Larkin & Scowcroft, 1981) can give rise to regenerants with new and potentially 'useful' characteristics and therefore can be considered to be an important technique in plant improvement (Heinz et al, 1977; Secor and Shepherd, 1981). With these facts in mind we have set out to find some of the causes and effects of variation within cultured cells and

regenerated plants of Capsicum frutescens Mill. Cv. annuum, the chilli pepper.

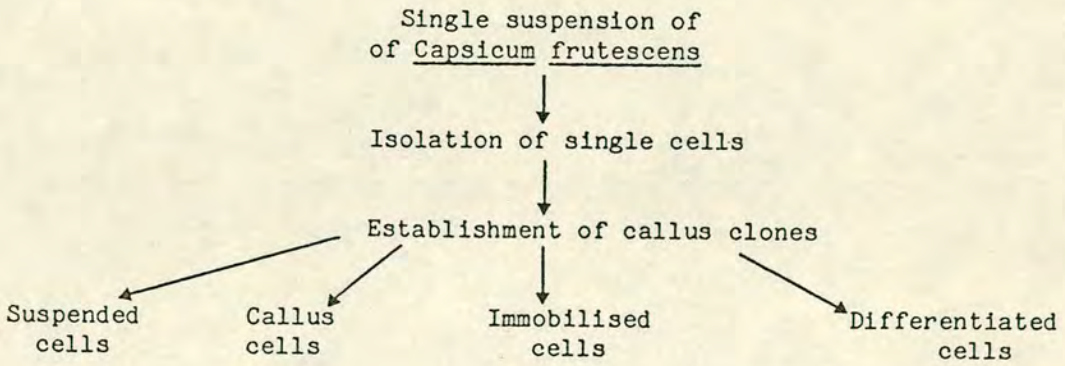
Clonal lines of Capsicum frutescens were established in order to examine interclonal variability and intraclonal stability with respect to various aspects of growth, structure and metabolic activity, namely, degree of friability, chlorophyll, protein, soluble phenolics, capsaicin and DNA contents. Comparisons were also made between these lines under different experimental regimes.

ISOLATION AND MAINTENANCE OF CLONES

Single cells were isolated from a single suspension culture from which three hundred callus cultures (clones) were established (Fig. 1). They were maintained, with a subculture every 4 weeks, on Schenk and Hildebrandt growth medium (Schenk & Hildebrandt, 1972) containing 0.5 mg l^{-1} 2,4-D, 2.0 mg l^{-1} pCPA, 0.1 mg l^{-1} kinetin, and 30 g l^{-1} sucrose. Investigations of the clones were made while maintained as callus, in suspension, as immobilised cells and as regenerated plants (Fig. 1)

Procedure for isolation of single cells A cell plating technique was used to isolate single cells using friable callus from a single suspension culture. Aggregates were sieved through a series of mesh filters down to 100uM in size, then centrifuged and diluted with conditioned medium to a volume of approx. 10^3 cells/ml^{-1}. They were then mixed with an equal volume of fresh medium and 1% low temperature agarose and plated out on a gridded Petri dish. The plated cells were examined microscopically for single cells which were marked so that their growth could be followed. When colonies were approx. 1mm in diameter they were pricked out and placed on 50% conditioned medium for one growth period and then transferred to normal SH medium. About 300 cell lines were established this way.

Figure 1. Isolation and maintenance of clones of Capsicum frutescens and their manipulation under differing environmental conditions.



VARIABILITY BETWEEN CLONES

Morphological differences Morphological differences are apparent as shown by the variation in the growth rates and friability of the clones (Table 1). Such differences affect the ability of the clones to immobilise in reticulate polyurethane. It can be seen that clones which are friable and contain little pigment have considerably higher growth rates (Table 1).

Metabolic differences Variation between clones is apparent in the chlorophyll and capsaicin contents (Table 1). Chlorophyll contents vary considerably and the most friable cultures tend to accumulate the lowest levels of chlorophyll (Table 1).

Table 1. Interclonal Variability Appearance and growth index, chlorophyll and capsaicin contents of selected clones of Capsicum frutescens.

Clone	Appearance	Growth Index	Chlorophyll/ Culture (µg)	Capsaicin/ Suspension culture (µg)
BV8	Green/Compact	1.35	15.0	1.08
N4	Yellow/friable	2.47	1.4	ND
CB6	Pale/Compact friable	3.85	2.4	0.028
B24	Yellow/Compact friable	2.21	1.8	ND
G3	Pale/Compact friable	0.57	2.9	3.56
CD5	Pale/Compact	0.08	2.8	0.1
AG2	Yellow/Green compact	1.36	12.91	1.15
Q5	Pale/friable	2.28	ND	ND
BQ1	Yellow/friable	0.06	ND	0.19
AN2	Pale/friable	1.32	ND	ND

ND = Not Detectable

Table 2. Interclonal Variability Apperance, growth index and capsaicin content from immobilised cultures of selected clones of Capsicum frutescens.

Clone	Appearance	Growth Index	Capsaicin/Immobilised Culture (µg)
AN2	Pale/friable	1.32	6.15
AE2	Yellow, green/ compact, friable	2.29	ND
F2	Yellow, green/ compact, friable	0.627	3.237
Z1	Pale green/friable	0.198	9.62
Y3	Pale green/friable	1.02	ND

$$\text{Growth Index} = \frac{\text{Final Weight} - \text{Initial Weight}}{\text{Initial Weight}}$$

Capsaicin production has been determined in suspended cell cultures (Table 1) and, in a small number of cases, in immobilised cell cultures (Table 2). Much variation is noticeable and in general cultures derived from callus with characteristically low growth rates tend to accumulate the highest yields (TABLES 1,2). In one instance immobilisation has resulted in the production of capsaicin, although the suspended clone failed to produce any detectable levels (AN2) and a second clone (Y3) was further found to begin production of dihydrocapsaicin on immobilisation. Each of the suspended clones examined was found to have a distinct profile of chloroform soluble compounds (mostly soluble phenolics) which accumulate in the liquid medium suggesting that each cell line has a unique metabolic finger print (Aitken & Yeoman 1986).

INSTABILITY OF THE CLONES

The clones show instability over a period of time as it can be seen that in some of the selected clones growth and appearance have changed over the 20 month maintenance period (Table 3). Clones which were initially slow growing with green compact growth habits were found to be stable in appearance and growth rate (Table 3). The initially faster growing clones, however, developed a reduced growth rate and became more compact and hard in structure (Table 3). It has been found that the morphologically more stable clones retained the capacity to produce capsaicin whereas the unstable ones accumulated increased level of phenolics but not capsaicin. There is also, in at least one case, evidence for the development of auxin habituation. Apart from an ability to grow in the absence of exogeneous auxin, cultures characteristically have an increased growth rate, increased friability, and decreased pigmentation and capsaicin contents.

TABLE 3. INTERCLONAL VARIABILITY Appearance and growth index of selected clones of Capsicum frutescens taken on two consecutive dates.

	August 1983		March 1985	
Clone	Appearance	Growth Index	Appearance	Growth Index
BV8	Green/compact	1.35	Hard, nodular	0.96
N4	Yellow/friable	2.47	Brown, compact	1.14
AE2	Yellow/friable compact	2.18	Yellow, friable	2.05
B24	Yellow/friable	2.21	Brown/compact	1.11
N3	Yellow/friable	1.09	Green/compact	0.54
CB6	Pale/compact friable	3.85	Pale, Brown/ compact	1.94
AG2	Yellow, green/friable	1.36	Dark/compact	0.44

VARIABILITY WITHIN CLONES

A fast growing and friable clonal line (AE2) was isolated to examine the amount of variation that occurs within a clone. An increasing number of isolates of the clone were maintained under the

three environmental regimes and analysis of growth and metabolic activity was made. It is apparent that there is variation in growth, protein and chlorophyll content within the clone (Table 4). It was noticeable that there were differences between the environmental regimes especially in the chlorophyll content of cells on agar and in suspension (Table 4). Work is being carried out to look at variation within immobilised cell cultures with regard to production of capsaicin and soluble phenolics.

Table 4 Intraclonal Variability Growth index, protein and chlorophyll contents of isolates of Clone AE2 of Capsicum frutescens on agar and in suspension media.

	CALLUS			SUSPENSION		
AE2 Replicates	Growth Index	Chlorophyll (µg/g FW)	Protein (mg/g FW)	Growth Index	Chlorophyll (µg/g FW)	Protein (mg/g FW)
1	3.97	0	2.56	4.49	0	1.55
2	7.86	16.9	2.4	7.83	12.55	1.6
3	8.94	33.38	2.58	9.39	7.14	1.08
4	8.35	4.31	1.35	6.41	12.49	1.5
5	13.45	10.68	1.34	6.75	10.58	1.88
6	8.71	40.87	2.38	7.77	10.55	1.6
7	14.85	4.84	1.92	8.39	11.04	1.73
8	10.70	14.52	1.56	8.22	-	1.2
9	14.63	15.62	2.15	8.34	-	1.6

Table 5 Stages of differentiation and regeneration associated with different ages and positions of explants from Capsicum frutescens

Explant (age)	Degree of Regeneration
Callus (18 months)	+
Callus (8 months)	+
Callus (4 months)	++
Leaf Disc (2 month plant)	++
Stem (2 month plant)	+++
Cotyledon/Hypocotyl	++++

Key:
+ Compaction: slowing of growth
++ Differentiation: greening
+++ Rooting or shooting
++++ Rooting and shooting

CLONE REGENERATION: VARIABILITY AMONG REGENERANTS

Work is in progress to develop a technique to regenerate the clones. It has been found that the period of time that cells are left in the 'undifferentiated' state is significant for successful

regeneration. A gradient of organogenic potential seems to exist with time in culture (Table 5). Therefore attempts to develop the necessary balance of growth regulators for regeneration has been prevented by the unstable state of the callus clones although aided by the use of physiologically younger hypocotyl and cotyledon material (Table 5). Work is being carried out to look at the stage at which the callus clones 'lose' their capacity to regenerate.

CONCLUSIONS AND FUTURE PROSPECTS

Preliminary results indicate that variability in morphological and biochemical markers is apparent between clones derived from a single suspension culture and that instability in these markers occurs over successive subcultures, particularly in initially rapidly-growing clones. The reasons for the variability and instability are not openly apparent and require further investigation. One approach being developed is to analyse changes in karyotype by an estimation of cellular DNA contents using fluorescent cell sorting techniques. A comprehensive examination of the effects of the environment - changes in pH, light, temperature - will be made to create the most favourable and stable conditions for growth. Instability does not necessarily mean a loss but rather a change in developmental potential and it is through an understanding of the nature of this change which will help make growth in vitro more stable and the manipulations of cultured tissues more accessible.

REFERENCES

Aitken, M.M. & Yeoman, M.M. (1986). The use of ELISA in cell line selection in tissue cultures of Capsicum frutescens. This volume 29.

Bayliss, M.W. (1980). Chromosomal variation in plant tissue in culture. Int. Rev. Cytol. Supply. 11A, 113-144.

Bennici, A., Buiatti, M. & D'Amato, F. (1968). Nuclear conditions in haploid Pelargonium in vitro and in vivo. Chromosoma 24, 194-201.

Dale, P.J. (1983). Protoplast culture and plant regeneration of cereals and other recalcitrant crops. Experientia Suppl. 46, 31-41.

Deus-Neumann, B. & Zenk, M.H. (1984). Instability of indole alkaloid production in Catharanthus roseus cell suspension cultures. Planta Medica 50, 427-431.

Heinz, D.J., Krishnamurthi, M., Nickell, L.G. & Maretzki, A. (1977). Cell tissue and organ culture in sugarcane improvement. In Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture, ed. J. Reinert and Y.P.S. Bajaj, pp. 3-17. Berlin and New York: Springer-Verlag.

Larkin, P.J. & Scowcroft, W.R. (1981). Somaclonal variation - a novel source of variability from cell cultures for plant improvement. Theor. Appl. Genet. 60, 197-214.

Lindsey, K. & Yeoman, M.M. (1985). Immobilised plant cell culture systems. In Primary and Secondary Metabolism of Plant Cell Cultures, ed. K.H. Neumann, W. Barz & E. Reinhard, pp. 304-315. Berlin Heidelberg: Springer-Verlag.

Meins, F. & Binns, A.N. (1977). Epigenetic variation of cultured somatic cells: Evidence for gradual changes in the requirement for factors promoting cell division. Proc. Natl. Acad. Sci. U.S.A. 74, 2928-2932.

Meins, F. (1983). Heritable variation in plant cell culture. Ann. Rev. Plant Physiol. 34, 327-346.

Mitra, J. & Steward, F.C. (1961). Growth induction in cultures of Haplopappus gracilis cells. IV. The behaviour of the nucleus. Am. J. Bot. 47, 358-68.

Renfroe, M.H. & Berlyn, G.P. (1985). Variation in nuclear DNA content in Pinus taeda L. Tissue cultures of diploid origin. J. Plant Physiol. 121, 131-39.

Scragg, A.H. & Fowler, M.W. (1985). The mass culture of plant cells. In Cell Culture and Somatic Cell Genetics of Plants, Vol. 2, ed. I.K. Vasil, pp. 103-128. London: Academic Press.

Secor, G.A. & Shepard, J.F. (1981). Variability of protoplast-derived potato clones. Crop Sci. 21, 102-105.

Schenk, R.U. & Hildebrandt, A.C. (1972). Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. 50, 199-204.

Skirvin, R.M. (1978). Natural and induced variation in tissue culture. Euphytica 27, 241-266.

Tabata, M. & Hiraoka, N. (1976). Variation of alkaloid production in Nicotiana rustica callus cultures. Physiol. Plant. 38, 19-23.

Thomas, E., King, P.J. & Potrykus, I. (1979). Improvement of crop plants via single cells in vitro - an assessment. Z. Pflanzenzüchtg. 82, 1-30.

Withers, L.A. (1983). Germplasm storage in plant biotechnology. In Plant Biotechnology, SEB Seminar Series, 18 eds. S.H. Mantell & H. Smith, pp. 187-218. London and New York: Cambridge Univ. Press.

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Manipulating Secondary Metabolism in Culture

VARIATION IN THE SECONDARY METABOLISM OF CULTURED PLANT CELLS.

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ABSTRACT

All plant cell cultures are heterogeneous, some more heterogeneous than others. The component cells may differ in size, structure, DNA content and in many other ways including metabolism. The proportion of cell types varies within and between cultures and changes with time. Indeed it is this continuous fluctuation in the cell population that has made it impossible to rely on the stability of the biosythetic activity of cultures. Nevertheless, although the decline in ability of a cell population to accumulate a designated product is commonly observed there are also examples where variation gives rise to cultures with an increased level of biosynthetic activity. It is therefore important to work towards an understanding of the underlying reasons for the pattern of spatial and temporal variation observed in tissue cultures for practical as well as theoretical reasons. In this paper an attempt has been made to examine temporal and spatial variation within a cell culture as well as within the cell population. Factors affecting these changes, namely, the heritable variation present in the explant and the influence of the culture conditions, are also discussed.

INTRODUCTION

The production of secondary metabolites by plant cell and issue cultures has commercial potential (Curtin, 1983; Tabata & ujita, 1985) as well as being useful in studying the biosynthesis and egulation of secondary products (Dougall, 1981). However, this otential of plant tissue cultures, both as a source of high value hemicals and as a system for studying secondary metabolism has not et been exploited mainly due to a lack of understanding of the roblems that arise as a result of culturing plant cells. Two such roblems are the variation and instability of the accumulation of econdary metabolites which frustrates attempts to maintain high and table yields in tissue culture as well as in large scale batch ultures (Fowler, 1986).

ariation arises as soon as tissues are excised from the donor plant nd placed in culture. Cell proliferation of this tissue rapidly eads to the development of a heterogeneous population of cells eoman and Forche, 1980). This heterogeneity is observed at the rphological (Davey et al, 1971), genetical (Meins & Binns, 1977;

Bayliss, 1980), molecular (McNay et al, 1984) and biochemical levels (Tabata and Hiraoka, 1976; Petiard et al, 1985; Guiller et al., 1987) and is known to increase with time in culture (Meins, 1983). It follows that these continual changes intrinsic to the culture process make it impossible to rely on the stability of metabolic activity or the production of secondary metabolites. Even the use of single cells or protoplasts to initiate cultures does not ensure the establishment of a homogeneous cell population, and continuous reselection is necessary to retain a desirable property of the culture (Ellis, 1985; Constabel et al, 1981). In this paper the problems of variation in tissue culture with particular reference to secondary metabolism will be considered.

Culture initiation and the effect of the donor plant

The variation found in a cell culture is strongly influenced by the explanted material from which it had been initiated. Excised explants are usually heterogeneous in nature consisting of a wide range of cell types which on proliferation lead to a complex cell population consisting of cells with differing growth rates, ploidy levels, physiology and other characteristics.

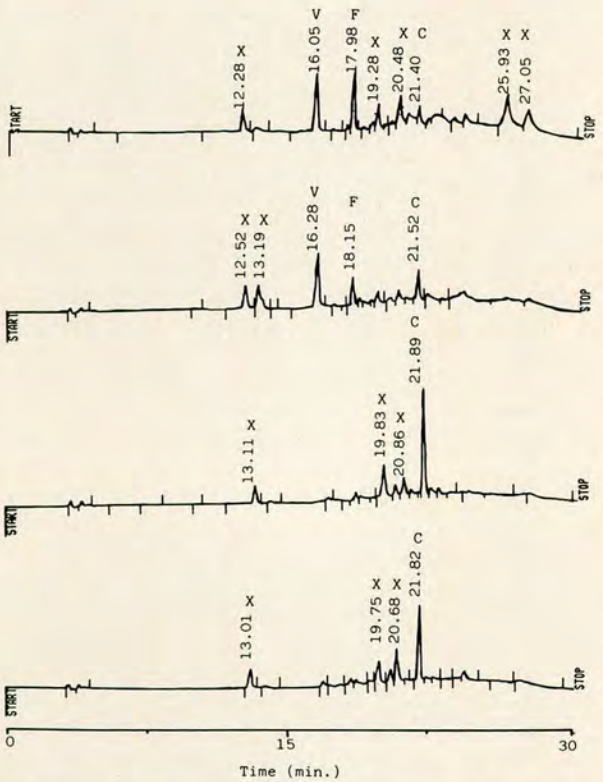
There is some evidence to suggest that the variation in biosynthetic activity observed in cultures is influenced by the intrinsic biosynthetic capacity of the explanted material. For example it has been shown that differences arise between cultures from explant material isolated from different donor plants; cultures derived from high yielding plants produce greater levels of secondary product than comparable cultures from low yielding plants (Zenk et al., 1977; Kinnersley and Dougall, 1980). This is not surprising since the production of a secondary metabolite is a genetically determined characteristic. Nevertheless, contrary to expectation, variation also arises in cultures derived from explants of the same plant, indeed it has been found in cultures of Ruta graveolens that those derived from the shoot region will only produce shoot-specific essential oils whereas those derived from the roots produce root-specific oils (Nagel & Reinhard, 1975). Similarly, isozyme patterns have been found to vary between suspension cultures derived from the root, cotyledon or hypocotyl of the same bean plant (Arnison & Boll, 1975). Therefore the donor plant can have a significant influence on variation in biosynthetic activity in culture through explants that are themselves heterogeneous; as well as having particular characteristics unique to the explant which can be passed on, at least during the initial stages of proliferation, to the resulting culture.

Intercultural Variation

In general it has been found that the biosynthetic activity of cell cultures is much lower than that of the donor plant (see for example Yeoman et al, 1980). Not only is there a drop in activity but also the production of secondary metabolites is very variable between cultures. Creche et al, (1987) have reported on the extent of variability in cultures of Choisya ternata obtained from two cell lines. They have shown that variation arises in the

roduction of two dihydrofuroquinoline alkaloids, platydesminium and alfourodinium, along with other morphological and physiological haracteristics. In this laboratory there is well documented evidence f differences between cultures of Capsicum frutescens, the chilli epper, with respect to the production of capsaicin as well as in ther biosynthetic properties (Holden et al, 1986). It has also een shown that the levels of three major precursors to capsaicin cinnamic acid, ferulic acid and vanillin) in the phenylpropanoid ranch of the capsaicin biosynthetic pathway has been found to vary etween isolates derived from one suspension culture (Fig. 1).

Fig. 1. HPLC chromatograms of extracts of chloroform-soluble phenolics taken from the medium of cell suspension isolates of Capsicum frutescens. Each chromatogram represents a different isolate derived from the one suspension culture.



The peaks represent the following; V = Vanillin, F = Ferulic Acid, C = Cinnamic Acid and X = Unknown phenolics. The respective retention times are given by each peak.

Intracultural variation

The differences found in the biosynthetic activity between cultures is a common phenomenon but difficult to explain. In the search for possible explanations it would seem logical to look to the source of these differences, that is, the culture itself. A single culture is known to be heterogeneous at any one time with respect to its biosynthetic activity (Dougall, 1987) and this type of variation has been described as intracultural or interspatial (Hall and Yeoman, 1986). To examine biosynthetic variation between cells within a single population two methods of analysis can be employed, indirectly by cloning single cells and assessing the resulting clonal culture or directly by an estimation of the cellular concentration of the designated product. Ideally both these procedures require cultures that synthesise stable and quantifiable amounts of a secondary product and, in the case of the latter, that remain within the cell after synthesis.

Table 1. Total capsaicin content (μg) of 10 suspended clones of *Capsicum frutescens*.

Clone no.	Capsaicin/ Suspension culture (μg)
BV8	1.08
N4	ND
CB6	0.028
B24	ND
G3	3.56
CD5	0.1
AG2	1.15
Q5	ND
BQ1	0.19
AN2	ND

ND = Not Detectable.

Assessment of intracultural variation by cloning

Cloning procedures have been used to isolate, or re-isolate high producing cell lines and in nearly every case there are reports of differences that arise between these cell lines with respect to biosynthetic activity (Dougall *et al*, 1980; Berlin *et al*, 1981; Deus-Neumann and Zenk, 1984; Dix, 1986). Tobacco cultures isolated by this procedure have been shown to vary considerably in their respective nicotine contents (Tabata and Hiraoka, 1976; Ogino *et al*, 1978). Likewise, cloning experiments using *Catharanthus roseus* cells have produced cultures that exhibit variation in their indole alkaloid contents; the range of yields of ajmalicine and serpentine were 0-0.85% and 0-1.4% per dry weight respectively (Zenk *et al*, 1977). It is debatable whether these cultures were always derived from single cells rather than from small cell aggregates due to the

difficulty of single cell isolation. Only a few reports have appeared describing true clonal cultures; suspension cultures of Anchusa officinalis established by clonal propagation of single cells were found to produce rosmarinic acid at different levels (Ellis, 1985). Investigations using protoclones, also of single 'cell' origin, have been carried out and have also shown differences in biosynthetic activity (Constabel et al., 1981; Nishimaki and Nozue, 1985; Petiard et al., 1985). Protoplasts have also been used to isolate high yielding 'cells' using flow cytometry, this technique is able to sort large populations of protopasts and isolate high producing cells from their fluorescence intensity (Brown, 1984).

Table 2. Growth and anthocyanin content of 26 cell lines of Catharanthus roseus and of the original stock culture. (Anthocyanin content measured in OD units per unit biomass per 10 cm^3 solvent.)

Cell line	Anthocyanin culture^{-1}	Anthocyanin g^{-1} fr. wt.	% Pigmented cells	Fresh weight (g)
109	3·18	1·01	20·2	3·17
103	1·46	0·70	15·3	2·07
013	1·45	0·34	5·8	4·30
201	1·40	0·59	14·9	2·37
002	1·20	0·46	11·9	2·65
006	1·19	0·49	10·3	2·59
008	1·16	0·37	10·5	3·11
018	1·04	0·28	11·2	3·74
104	1·00	0·36	11·0	2·73
108	0·85	0·27	5·9	3·18
012	0·85	0·35	10·8	2·36
001	0·80	0·30	9·7	2·71
007	0·79	0·31	6·8	2·58
010	0·79	0·31	8·3	2·57
101	0·61	0·30	9·2	2·04
011	0·56	0·11	3·0	3·87
009	0·53	0·15	2·9	3·57
021	0·46	0·17	4·7	2·74
015	0·37	0·14	3·8	2·54
107	0·26	0·08	1·9	3·26
003	0·23	0·08	2·7	2·75
018	0·23	0·09	2·7	2·62
105	0·17	0·07	2·1	2·58
106	0·15	0·04	0·8	3·55
017	0·14	0·04	1·2	3·49
014	0·10	0·03	0·6	3·40
l.s.d.	0·26	0·07	—	0·46
Stock	1·43	0·42	9·1	3·39

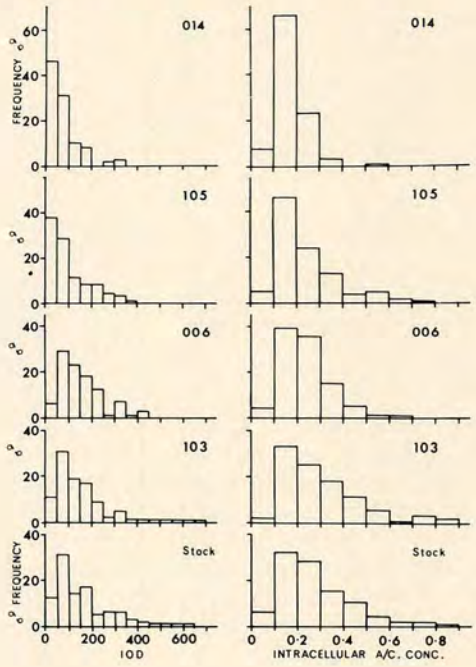
In this laboratory cloning experiments have been carried out using Capsicum frutescens and Catharanthus roseus cultures (Holden et al., 1986; Hall and Yeoman, 1987). Pepper cultures derived from aggregate clones have been shown to vary considerably in their capacity to produce capsaicin (Table 1). Similarly anthocyanin content was found to vary >30 fold in twenty-six clones isolated from single cells of a Catharanthus roseus culture (Table 2). There was also a positive correlation between these differences in anthocyanin yield and the proportion of pigmented (anthocyanin producing) cells within each clone (Table 2). Here it is interesting to note that all these clones were capable of anthocyanin synthesis despite the fact that they had been isolated from a culture in which only 10% of the population accumulated anthocyanin (Hall and Yeoman, 1987).

These reports show that cultures derived from a single cell, protoplast or aggregate of the same culture exhibit differences in biosynthetic activity. Nevertheless, although this would suggest that the parent culture was biosynthetically heterogeneous, we should not ignore the variation that will inevitably arise during the time of clone establishment, and therefore a more direct method should be used that eliminates this temporal effect.

Assessment of intracultural variation using microdensitometry

Microspectrophotometric or microdensitometric techniques can be used to assess the intracellular concentration of a designated product within a single cell in a population. Therefore the variation in biosynthetic activity can be determined between cells within a culture at one time therefore eliminating any temporal effects. Microspectrophotometric analysis of the intracellular concentration of rosmarinic acid in single cells of an Anchusa culture has revealed great differences between individual cells (Chaprin and Ellis, 1984). In this laboratory microdensitometric determinations of individual cells of the Catharanthus cell lines (Table 2) has also shown significant amounts of intracellular variation (Fig. 2). It was also found that although the differences

Fig. 2. Variation in intracellular anthocyanin content (left: IOD = integrated optical density) and intracellular anthocyanin concentration (right: IOD/cell volume) in light-grown callus cultures of four Catharanthus roseus cell lines and the original stock culture.



in content were great between cells there were no significant differences in the mean intracellular anthocyanin values between cell lines (Table 3). This suggests that the 30 fold differences in anthocyanin content found between cell lines were attributable to the number of productive cells and not to the differences in the intracellular anthocyanin content (Table 2, Fig. 2). Therefore this would suggest that, as all of the cells within a culture are capable of producing anthocyanin, only a proportion of them do so at a particular time. The ultimate goal would be to create conditions that would 'induce' more cells to be productive, bringing about higher and, hopefully, more stable yields of the designated product.

Table 3. Microdensitometric analysis of the visibly pigmented cell populations of C. roseus within late stationary phase callus cultures (light grown) of four cell lines and the original stock culture.

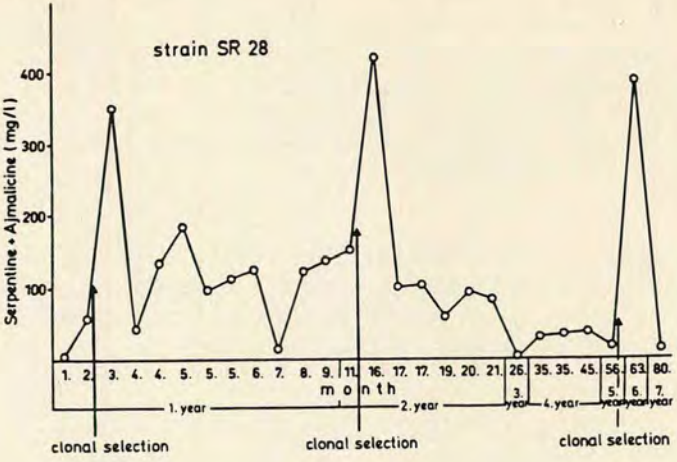
Cell line	IOD[a]	Range	IOD/vol[b]	Range
014	76·7 ± 6·8	8–337 (42×)	0·176 ± 0·007	0·07–0·53 (7×)
105	100·4 ± 8·9	8–377 (45×)	0·239 ± 0·014	0·08–0·75 (9×)
006	149·9 ± 9·4	12–465 (38×)	0·229 ± 0·011	0·09–0·69 (8×)
103	144·6 ± 10·7	18–614 (34×)	0·297 ± 0·017	0·08–0·87 (11×)
Stock	161·0 ± 14·1	14–645 (46×)	0·247 ± 0·015	0·10–0·79 (8×)

[a] IOD is equivalent to the mean anthocyanin content/cell.
[b] IOD/vol is equivalent to the mean intracellular anthocyanin concentration.

Instability of production: temporal variation

As well as showing indirectly the degree of intraclonal variation the use of cell line selection or cloning of high producing cells is a way of establishing high yielding cultures (Zenk and Deus, 1982; Sato and Yamada, 1984; Dix, 1986). Although at present this procedure has only been used with cells that produce pigments that can be readily identified and quantified, for example anthocyanins and carotenoids, cultures with product yields greater than that of the parent plant have been established (Zenk et al., 1977). Nevertheless, the isolation of high producing lines that are stable over time are difficult to establish, in most cases the production falls. Catharanthus roseus cultures isolated from high yielding cells have shown a considerable amount of biochemical instability by showing a drop in the production of ajmalicine and serpentine with time (Zenk et al., 1977). In fact it is only by continuous reselection of high producing cells that the high yielding properties can be retained. As we can see in Fig. 3 clonal selection raises the serpentine and ajmalicine contents of a Catharanthus roseus strain significantly, but this level is not maintained for it rapidly drops to a low value again (Deus-Neumann & Zenk, 1984).

Fig. 3. Production behaviour of Catharathus roseus strain SR28 during a period of seven years with a total of three clonal selections. (From Deus-Neumann and Zenk, 1984).



Evidence of biochemical instability has also been found elsewhere with tissue cultures. Tabata and Hiraoka (1976) showed that the nicotine content of tobacco callus either from the seed, root or leaf of Nicotiana rustica dropped to a very small amount by the 8-9th culture passage. Similarly, callus cultures of Digitalis purpurea lose the ability to produce certain cardenolides with time in culture (Hirotani and Furuya, 1977). In this laboratory it has been shown that Capsicum suspension cultures show biochemical instability with respect to the production of cinnamic acid (Fig. 4). However, in this case the levels of precursor increased by the end of a sixteen week period in suspended isolates (Fig. 4). Nevertheless, biochemical instability is not always the rule. It has been shown that cultures producing high yields of nicotine by cell line selection in tobacco maintained their high yielding capacity over a period of twelve months without further selection (Ogino et al, 1976). In this laboratory it has been shown that although there was wide variation in the capacity of Catharanthus cell lines to produce anthocyanins this production was nevertheless stable over a 5-6 month period (Fig. 5). (Hall and Yeoman, 1987).

Factors affecting temporal variation

Factors that affect the increase or decrease in the biosynthetic activity of cell cultures are of interest to both those trying to understand variation as well as to workers looking for high yields of secondary metabolites. If cells are exposed to the appropriate cultural stimuli then it might be possible to produce cultures with a significant and stable yield of product. Such stimuli can either come from the physical environment, for example by light (Knobloch et al., 1982) or temperature (see review by Mantell and Smith, 1983) or from the components of the medium. It is the latter

Fig. 4. Total cinnamic acid content (µg) found in the medium of four suspended culture isolates of Capsicum frutescens over a sixteen week period.

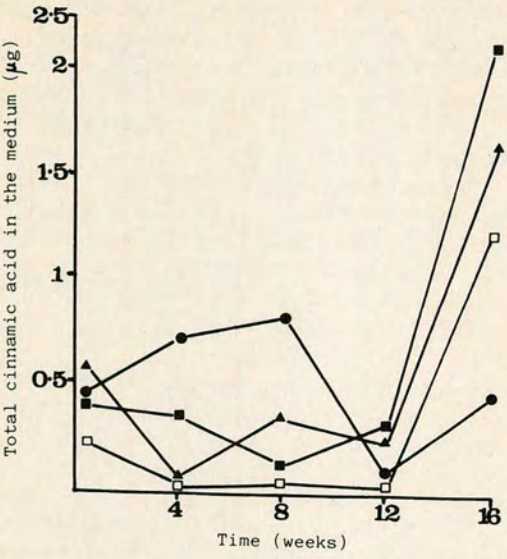
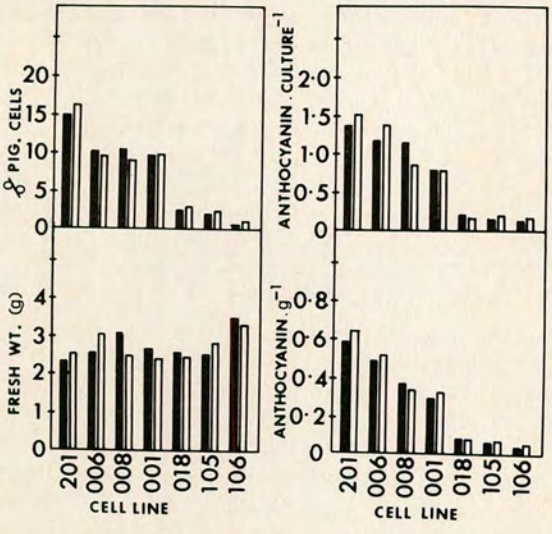


Fig. 5. The stability of culture growth and anthocyanin production in selected cell lines of Catharanthus roseus when grown in the light.



The results presented as unshaded bars were obtained c. 6 months after those which are shaded. (Anthocyanin content measured in OD units per unit biomass per 10 cm^3 solvent)

that appears to have the greatest effect on product yield, and will now be discussed.

Plant growth regulators can be effective in stimulating or inhibiting secondary product synthesis in cell cultures. However, they are used in the growth media primarily to induce and establish callus from an explant and to maintain proliferation (Yeoman and Forche, 1980). Auxins, rather than cytokinins, appear to have the greatest influence on biosynthetic activity (Dougall, 1980). The auxin, 2,4-D has been shown to inhibit nicotine production in tobacco cells (Tabata and Hiraoka, 1976) although another auxin, IAA, caused tobacco cultures to produce nicotine as well as anatabine and anabasine (Furuya *et al.*, 1971). Other auxins, like NAA, have also been shown to have promotory characteristics. Cells of *Morinda citrifolia* grown in the presence of NAA showed a substantial production of anthraquinones, while those grown with 2,4-D did not (Zenk *et al.*, 1975).

Other components of the medium are known to affect the biosynthetic activity of cultured cells. Nutrient composition, mainly the levels of nitrate, phosphate, ammonia and potassium are known to affect both the primary and secondary metabolism of cultured plant cells (see review, by Dougall, 1980). Knobloch and Berlin (1983) have shown that a rapid accumulation of secondary compounds occurs when cells of *Catharanthus roseus* were grown in a medium devoid of phosphate. This result has also been demonstrated with carrot (Dougall and Weyrauch, 1980) and sycamore cultures (Phillips and Henshaw, 1977). Similarly media with reduced levels of nitrate and ammonia appear to stimulate biosynthetic activity (Phillips and Henshaw, 1977). Lindsey (1985) has shown that a reduction in the nitrate and phosphate levels of the medium leads to a reduction in the incorporation of ^{14}C Phenylalanine into protein and an increase in the incorporation of this precursor into capsaicin in pepper cultures.

The concentration of the carbon source in the medium is also known to affect secondary metabolism. Increased sucrose levels have been shown to stimulate phenolic synthesis and PAL activity in cultured sycamore cells (Westcott and Henshaw, 1976; Phillips and Henshaw, 1977). Similarly high sucrose concentrations stimulated alkaloid synthesis in *Catharanthus roseus* cultures (Knobloch *et al.*, 1982).

From this it would appear that the media components play an important role in the control of the biosynthetic activity of cultured cells. As these components are used in varying amounts in different media and the levels will fluctuate considerably during the culture cycle it is inevitable that this will give rise to biosynthetic variation. Nevertheless it is by the strict regulation of components of the medium at all stages of culture, that high levels of variation and stability in biosynthetic activity may be controlled.

CONCLUDING REMARKS

Variation in biosynthetic activity arises in cell cultures and can result in either a decrease or increase in product yield. It is common to all cultures as well as within a culture, and is expressed over a growth cycle and generally increases over successive growth cycles. It is also expressed within an individual cell and this creates difficulties when biosynthetically active cells are being isolated by cloning techniques.

Variation in biosynthetic activity is influenced by the origin and type of explant from which the culture is initiated and by the culture conditions. Although influenced by heritable and environmental factors, culture variation is a direct result of genomic instability. There is well documented evidence of chromosomal changes and point mutations occurring within cells of plant cultures (Bayliss, 1980; Meins, 1983). But not all variation in cell cultures is attributable to these gross rearrangements of cell karyotype, there are also highly frequent, reversible changes in gene expression, epigenetic changes, which are just as important (Meins, 1983). It is these epigenetic changes that are more likely to be associated with the control and regulation of the biosynthetic activity of cultured cells. Plant secondary biosynthetic pathways *in vivo* or *in vitro* are highly regulated systems. The level of production of a particular secondary product will be influenced by the availability of precursors and the activity of specific pathway enzymes. More importantly, the activity of these enzymes will be controlled by gene or multi-gene expression. Therefore the switching 'on' or 'off' of these pathway-related genes will have profound effects on the metabolism of secondary products. It is this epigenetic control of synthesis that could explain why the *Catharanthus* cultured plant cells in our laboratory all have the capacity to produce anthocyanin but are not always able to do so, and why there is so much variation within a culture with respect to the number of productive and non-productive cells. Future work should look at biosynthetic variation at the level of gene expression so a tighter control on variation can be made possible. Therefore an understanding of what 'triggers' the increase in the number of producing cells would lead not only to increased yields but also to maintain enhanced levels of stability.

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REFERENCES

Arnison, P.G. and Boll, W.G. (1975). Isoenzymes in cell cultures of bush bean (*Phaseolus vulgaris* cv. Contender): isoenzymatic differences between stock suspension cultures derived from a single seedling. Can. J. Bot., 53, 261-71.

Bayliss, M.W. (1980). Chromosomal variation in plant tissues in culture. Int. Rev. Cytol. Suppl., 11A, 113-44.

Berlin, J., Kukoschke, K.G. and Knobloch, K.-H. (1981). Selection of tobacco cell lines with high levels of cinnamoyl putrescines. Planta Med., 42, 173-80.

Brown, S. (1984). Analysis and sorting of plant material by flow cytometry. Physiol. Veg., 22, 341-49.

Chaprin, N. and Ellis, B.E. (1984). Microspectrophotometric evaluation of rosmarinic acid accumulation in single cultured plant cells. Can. J. Bot., 62, 2278-82.

Constabel, F., Rambold, S., Chatson, K.B., Kurz, W.G.M. and Kutney,J.P. (1981). Alkaloid production in Catharanthus roseus (L.) G.Don. VI. Variation in alkaloid spectra of cell lines derived from one single leaf. Plant Cell Reports, 1, 3-5.

Creche, J., Guiller, J., Andreu, F., Gras, M., Chenieux, J.-C. and Rideau, M. (1987). Variability in tissue cultures of Choisya ternata originating from a single tree. Phytochemistry, 26, 1947-53.

Curtin, B. (1983). Harvesting profitable products from plant tissue culture. Bio/Technology, 1, 649-57.

Davey, M.R., Fowler, M.W. and Street, H.E. (1971). Cell clones contrasting in growth, morphology and pigmentation isolated from a callus culture of Atropa belladonna var. Lutea. Phytochemistry, 10, 2559-75.

Deus-Neumann, B. and Zenk, M.H. (1984). Instability of indole alkaloid production in Catharanthus roseus cell suspension cultures. Planta Med., 50, 427-31.

Dix, P.H. (1986). Cell line selection. In Plant Cell Culture Technology, ed. M.M. Yeoman, pp. 143-201. Botanical Monographs, Vol. 23. Blackwell Scientific Publications.

Dougall, D.K. (1980). Nutrition and metabolism. In Plant Tissue Culture as a Source of Biochemicals, ed. E.J. Staba, pp. 21-58. CRC Press, Boca Raton, Florida.

Dougall, D.K. (1981). Tissue culture and the study of secondary natural products. In The Biochemistry of Plants: Secondary Plant Products, Vol. 7, ed. E.E. Conn, pp.21-34. Academic Press, New York.

Dougall, D.K. (1987). Cell cloning and the selection of high yielding strains. In Cell Culture and Somatic Cell Genetics of Plants, Vol. 4, eds. F. Constabel and I.K. Vasil, pp. 117-24. Academic Press, New York.

Dougall, D.K., Johnson, J.M. and Whitten, G.H. (1980). A clonal analysis of anthocyanin accumulation by cell cultures of wild carrot. Planta, 149, 292-97.

Dougall, D.K. and Weyrauch, K.W. (1980). Growth and anthocyanin production by carrot suspension cultures grown under chemostat conditions with phosphate as the limiting nutrient. Biotech. Bioeng., 25, 581-94.

Ellis, B.E. (1985). Characterisation of clonal cultures of Anchusa officinalis derived from single cells of known productivity. J. Plant Physiol., 119, 149-58.

Fowler, M.W. (1986). Industrial applications of plant cell culture. In Plant Cell Culture Technology, ed. M.M. Yeoman, pp. 202-27. Botanical Monographs, Vol. 23. Blackwell Scientific Publications.

Furuya, T., Kojima, H. and Syono, K. (1971). Regulation of nicotine biosynthesis by auxins in tobacco callus tissues. Phytochemistry, 10, 1529-32.

Guiller, J.T., Andreu, F., Creche, J., Chenieux, J.-C. and Rideau, M. (1987). Variability in tissue cultures of Choisya ternata. Alkaloid accumulation in protoclones and aggregate clones obtained from established strains. Plant Cell Reports, 6, 375-78.

Hall, R.D. and Yeoman, M.M. (1986). Temporal and spatial heterogeneity in the accumulation of anthocyanins in cell cultures of Catharanthus roseus (L). G. Don. J. Exp. Bot., 37, 48-60.

Hall, R.D. and Yeoman, M.M. (1987). Intercellular and intercultural heterogeneity in secondary metabolite accumulation in cultures of Catharanthus roseus following cell line selection. J. Exp. Bot., 38, 1391-98.

Hirotani, M. and Furuya, T. (1977). Restoration of cardenolide synthesis in redifferentiated shoots from callus cultures of Digitalis purpurea. Phytochemistry, 16, 610-11.

Holden, P.R., Aitken, M., Lindsey, K. and Yeoman, M.M. (1986). Variability and stability of cell cultures of Capsicum frutescens. In Secondary Metabolism of Plant Cell Cultures, eds. P. Morris, A.H. Scragg, A. Stafford and M.W. Fowler, pp. 237-43. Cambridge: Cambridge University Press.

Kinnersley, A.M. and Dougall, D.K. (1981). Correlation between the nicotine content of tobacco plants and callus cultures. Planta, 149, 205-6.

Knobloch, K.-H., Bast, G. and Berlin, J. (1982). Medium- and light-induced formation of serpentine and anthocyanins in cell suspension cultures of Catharanthus roseus. Phytochemistry, 21, 591-4.

Knobloch, K.H. and Berlin, J. (1983). Influence of phosphate on the formation of the indole alkaloids and phenolic compounds in cell suspension cultures of Catharanthus roseus. Plant Cell and Organ Culture, 2, 333-40.

Lindsey, K. (1985). Manipulation, by nutrient limitation, of the biosynthetic activity of immobilized cells of Capsicum frutescens. Planta, 165, 126-33.

Mantell, S.H. and Smith, H. (1983). Cultural factors that influence secondary metabolite accumulations in plant cell and tissue cultures. In Plant Biotechnology, eds. S.H. Mantell and H. Smith, pp. 75-110. Cambridge: Cambridge University Press.

Meins, F. and Binns, A. (1977). Epigenetic variation of cultured somatic cells: evidence for gradual changes in the requirement for factors promoting cell division. Proc. Nat. Acad. Sci. USA, 74, 2928-32.

Meins, F. (1983). Heritable variation in plant cell culture. Ann. Rev. Plant Physiol., 34, 327-46.

McNay, J.W., Chourey, P.S. and Pring, D.R. (1984). Molecular analysis of genomic stability of mitochondrial DNA in tissue cultured cells of Maize. Theor. Appl. Genet., 67, 433-37.

Nagel, M. and Reinhard, E. (1975). Das Aetherische Del der Calluskulturen von Ruta graveolens L. Die Zugammensetzung des Oeles. Planta Med., 27, 151-58.

Nishimaki, T. and Nozue, M. (1985). Isolation and culture of protoplasts from high anthocyanin-producing callus of sweet potato. Plant Cell Reports, 4, 248-51.

Ogino, T., Hiraoka, N. and Tabata, M. (1978). Selection of high nicotine-producing cell lines of tobacco by single-cell cloning. Phytochemistry, 17, 1907-10.

Petiard, V., Baubault, C., Bariaud, A., Hutin, M. and Courtois, D. (1985). Studies on variability in plant tissue cultures for alkaloid production in Catharanthus roseus and Papaver somniferum callus cultures. In Primary and Secondary Metabolism of Plant Cell Cultures, eds. K.-H. Neumann, W. Barz and E. Reinhard, pp. 133-42. Springer-Verlag, Berlin and New York.

Phillips, R. and Henshaw, G.G. (1977). The regulation of synthesis of phenolics in stationary phase cell cultures of Acer pseudoplatanus L. J. Exp. Bot., 28, 785-94.

Sato, F. and Yamada, Y. (1984). High berberine-producing cultures of Coptis japonica cells. Phytochemistry, 23, 281-5.

Tabata, M. and Hiraoka, N. (1976). Variation in alkaloid production in Nicotiana rustica callus cultures. Physiol. Plant., 38, 19-23.

Tabata, M. and Fujita, Y. (1985). Production of shikonin by plant cell cultures. In Biotechnology in Plant Science: Relevance to Agriculture in the Eighties, eds. M. Zaitlin, P. Day and A. Hollaender, pp. 207-218. Academic Press, New York.

Westcott, R.J. and Henshaw, G.G. (1976). Phenolic synthesis and phenylalanine ammonia-lyase activity in suspension cultures of Acer pseudoplatanus L. Planta, 131, 67-73.

Yeoman, M.M. and Forche, E. (1980). Cell proliferation and growth in callus cultures. Int. Rev. Cytol. Suppl., 11A, 1-24.

Yeoman, M.M., Miedzybrodzka, M.B., Lindsey, K. and McLauchlan, W.R. (1980). The synthetic potential of cultured plant cells. In Plant Cell Cultures: Results and Perspectives, eds. F. Sala, B. Parisi, R. Cella and O. Cifferi, pp. 327-43. Elsevier-North Holland, Amsterdam.

Zenk, M.H., El-Shagi, H. and Schulte, V. (1975). Anthraquinone production by cell suspension cultures of Morinda citrofolia. Planta Med. Suppl., 79-101.

Zenk, M.H., El-Shagi, H., Arens, H., Stockigt, J., Weiler, E.W. and Deus, B. (1977). Formation of the indole alkaloids serpentine and ajmalicine in cell suspension culture of Catharanthus roseus. In Plant Tissue Culture and Its Biotechnological Application, eds. W. Barz, E. Reinhard and M.H. Zenk, pp.27-43. Springer-Verlag, Berlin and New York.

Zenk, M.H. and Deus, B. (1982). Natural product synthesis by plant cell cultures. In Plant Tissue Culture, 1982, ed. A. Fujiwara, pp. 391- 394. IAPTC, Tokyo.

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Manipulating Secondary Metabolism in Culture

THE EFFECTS OF FUNGAL ELICITATION ON SECONDARY METABOLISM IN CELL CULTURES OF CAPSICUM FRUTESCENS.

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ABSTRACT

Stationary phase suspension cultures of Capsicum frutescens were elicited by a range of concentrations of spores of a pathogenic fungus Gliocladium deliquescens for a 24-hour period prior to the addition of ^{14}C Cinnamic Acid a radio-active precursor to Capsaicin . Analysis of the $CHCl_3$ soluble component of the medium (mostly unknown phenolics, major precursors to capsaicin, and capsaicin) harvested 24 hours after the addition of the label was carried out using HPLC techniques, fractionation and scintillation spectroscopy. Results show that elicitation alters the pattern of phenolic production and increases the incorporation of label into capsaicin and its precursors. This alteration in flux not only increases capsaicin yield but presents more evidence on the control and regulation of Capsaicin biosynthesis.

INTRODUCTION

The accumulation of capsaicin (the pungent principle of the chilli pepper fruit) in cell cultures of Capsicum frutescens has proved a useful experimental system to study the regulation of secondary metabolite production (Yeoman et al., 1980). Previous investigations have shown that suspension cultures produce significantly less capsaicin than the fruit (Hall et al., 1987). However manipulation of culture conditions through e.g. nutrient limitation, cell immobilization and precursor feeding have increased capsaicin yields to levels greater than those found in the fruit (Lindsey and Yeoman, 1984). These procedures have also helped in understanding the control and regulation of capsaicin synthesis (Hall et al., 1987; Holden et al., 1987).

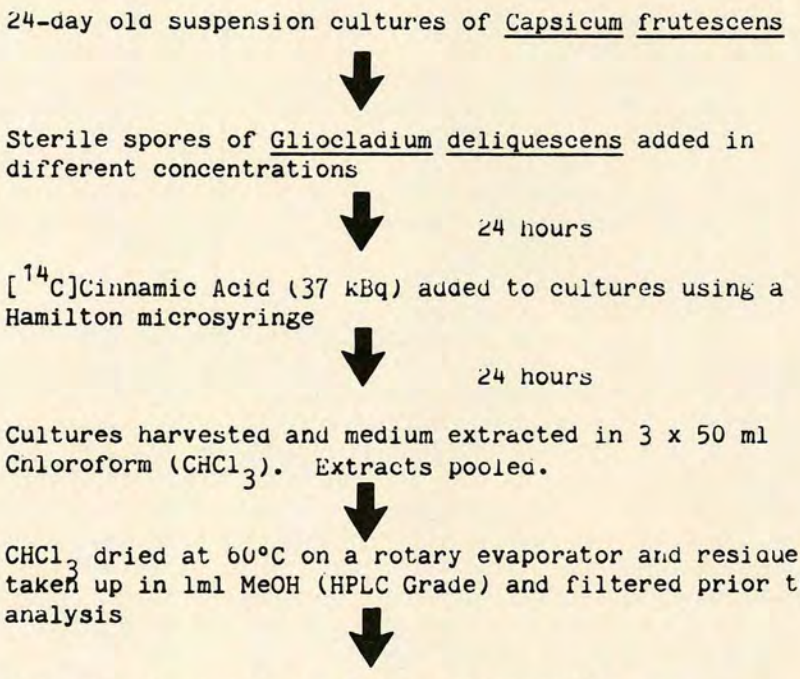
Recently we have adopted an additional approach to facilitate studies on capsaicin synthesis by using elicitors. It is well known that fungal elicitation using a range of preparations from pathogenic and non- pathogenic organisms will raise the yield of certain secondary metabolites in cell cultures (Hahlbrock et al., 1981; Funk et al., 1987) by increasing the activity of specific enzymes in pathways to these metabolites (Dixon et al., 1986). In this study the effects of a fungal elicitor fed to cell cultures of the chilli pepper on the incorporation of a radioactive precursor into phenolics and capsaicin has been investigated.

MATERIALS AND METHODS

Cell cultures

Suspension cultures of Capsicum frutescens Mill. cv. annuum, derived from stem callus, were maintained in 250 ml Erlenmeyer flasks in 50 ml of liquid Schenk and Hildebrandt medium (Schenk and Hildebrandt, 1972) supplemented with 0.5 mgl^{-1} 2,4-D, 0.1 mgl^{-1} kinetin, 2.0 mgl^{-1} pCPA and 30 gl^{-1} sucrose. Cells were agitated on a rotary shaker (98 rev. min^{-1} with 0.8 cm amplitude) at 25±1°C in continuous light (Compton warmwhite) at an irradiance of 25 $\mu molm^{-1}$, and were sub-cultured every 28 days. Cells cultured for 24 days were used in the experiments described.

Fig. 1. Plan of experiment and fate of radioactive precursor ^{14}C Cinnamic Acid.



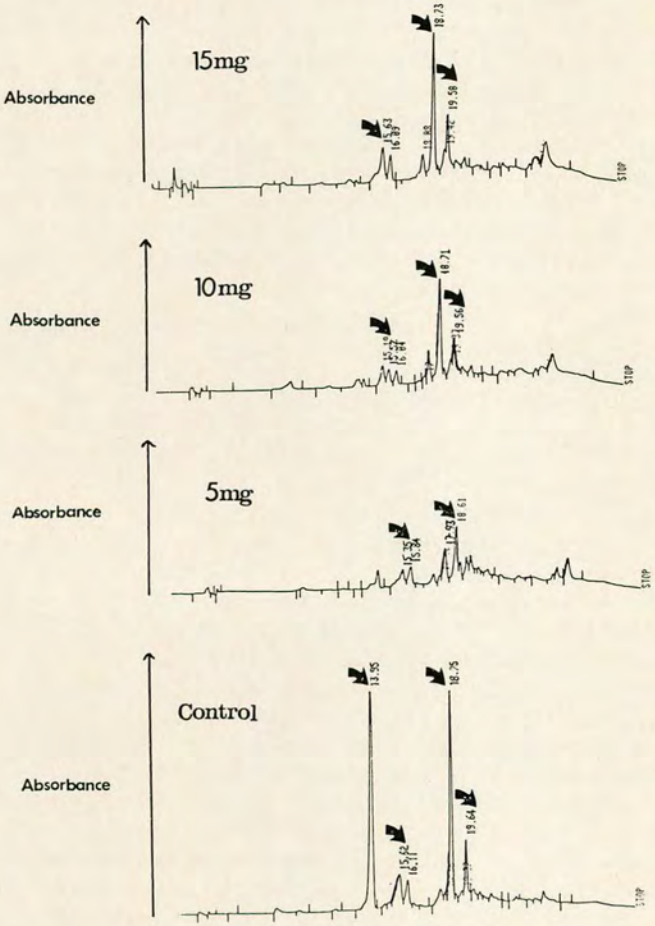
Spore preparation

Spores of the fungus Gliocladium deliquescens were inoculated on-to the full growth medium of Schenk and Hildebrandt (see above) that was solidifed with 0.6% Agar. The fungus was cultured for 14 days, after which the spores were washed off with distilled water, centrifuged at 2,000 g, freeze-dried and then autoclaved before use. Aliquots of spores of 5, 10, 20, 25 and 30 mg/ per flask were added by automatic pipette to cell cultures 24 hours before addition of the radioactive precursor (Fig. 1).

Radioactive labelling

Sterile aliquots (2μCi, 40μl) of [^{14}C]Cinnamic Acid (Amersham plc) were added directly to the sterile culture medium (Fig. 1) and a labelling period of 24 hours was used throughout. Radioactivity incorporated into phenolic intermediates (major phenolics to capsaicin) and capsaicin was determined using liquid scintillation spectrometry.

Fig. 2. Chromatograms of extracts of $CHCl_3$ soluble phenolics taken from the medium of cultures receiving 0,5,10,15 mg/flask of spores of Gliocladium deliquescens. Arrowed peaks indicate major phenolics.



Extraction and analysis

The medium was extracted three times with 50ml of $CHCl_3$ and the extracts pooled (Fig. 1). After drying *in vacuo* at 60°C the residue was taken up in 1ml HPLC grade MeOH and filtered prior to

chromatography using a Hewlett-Packard HP1090 Liquid Chromatograph fitted with a gradient pumping system and a HP1040 Diode Array Detector. Separation was carried out at 40°C using a 200 x 5mm Hypersil MOS column on a gradient using MeOH and 5% aq. acetic acid as the eluting solvent. All the major precursors to capsaicin as well as several unknown phenolics were separated in a single 30 min run and each peak was identified by its characteristic spectrum.

RESULTS

The elicitation of 24 day old cell suspension cultures of Capsicum frutescens using a concentration range of sterile spores of Gliocladium deliquescens appears to alter the pattern of $CHCl_3$ soluble phenolics in the culture medium (see Fig. 2). Increasing the amount of elicitor appears to increase the levels of major phenolics although, in general, treated cells produce less of these major phenolics than the untreated cells. Capsaicin and its major precursors are not being produced in sufficient quantities to be identified on these chromatographs (Fig. 2). Also elicitation increased the incorporation of ^{14}C Cinnamic Acid into the pathway intermediates (major phenolic precursors) and into capsaicin present in the medium (Figs. 3, 4). Indeed there is a positive correlation between incorporation into the intermediates of the capsaicin pathway and the concentration of added elicitor, although the treatment with the lowest amount of elicitor displays a lower level of incorporation than the control (Fig. 3). The incorporation of ^{14}C Cinnamic Acid into capsaicin in the medium does not show a progressive increase over this elicitor range although there is a significant increase in incorporation into capsaicin with the highest amount of elicitor (Fig. 4).

DISCUSSION

Elicitation of cell suspension cultures of Capsicum frutescens alters the amount of total $CHCl_3$ soluble phenolics in the medium after a 24 hour labelling period. This difference which presumably reflects an alteration in secondary metabolism can be attributed to the production of capsaicin and capsaicin pathway intermediates (Phenolics) in the medium with increased levels of elicitation as shown in the labelling experiments. Therefore elicitation does appear to alter the flow of metabolites down the capsaicin biosynthetic pathway and treatments with high amounts of elicitor direct more radioactive precursor into capsaicin, the final product. Results obtained recently in this laboratory suggest one contributing factor to this increase is the stimulatory effect of these spores on Phenylalanine Ammonia Lyase (PAL), a major enzyme in the pathway.

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Fig. 3
Incorporation (Dpm) of ^{14}C Cinnamic Acid into free intermediate phenolic component (major precursors) of the medium with or without the addition of elicitor. 6 x 10^5 counts represent 30% of the total label incorporated.

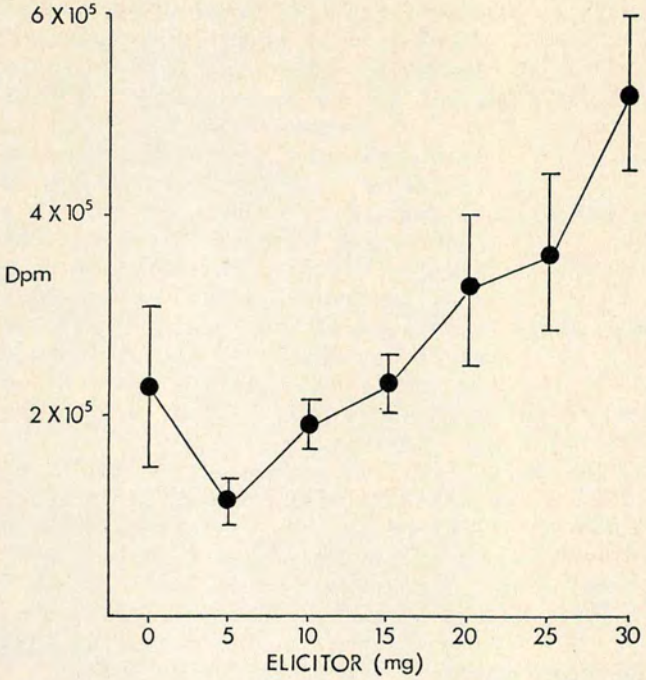
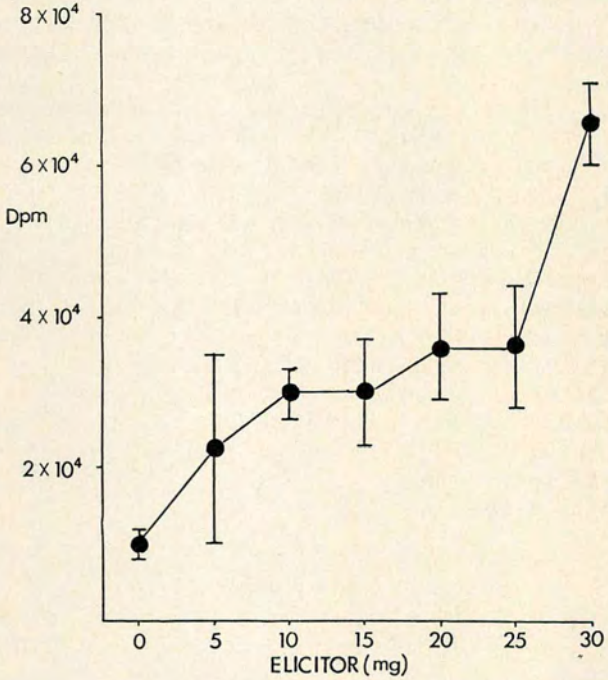


Fig. 4.
Incorporation (Dpm) of ^{14}C Cinnamic Acid into ...psaicin in the medium ...h or without the ...ddition of elicitor. ...3 x 10^4 represents 4% of the total label incorporated.



REFERENCES

Dixon, R.A., Bailey, J.A., Bell, J.N., Bolwell, G.P., Cramer, C.L., Edwards, K., Hamdan, M.A.M.S., Lamb, C.J., Robbins, M.P., Ryder, T.B. and Schuch, W. (1986). Rapid changes in gene expression in response to microbial elicitation. Phil. Trans. R. Soc. Lond. B314, 411-426.

Funk, C., Gugler, K. and Brodelius, P. (1987). Increased secondary product formation in plant cell suspension cultures after treatment with a yeast carbohydrate preparation (Elicitor). Phytochemistry 26, 401-405.

Hahlbrock, K., Lamb, C.J., Purwin, C., Ebel, J., Fautz, E. and Schafer, E. (1981). Rapid response of suspension-cultured parsley cells to the elicitor from Phytophora megasperma var. sojae. Plant Physiology 67, 768-773.

Hall, R.D., Holden, M.A. and Yeoman, M.M. (1987). The accumulation of phenylpropanoid and capsaicinoid compounds in cell cultures and whole fruit of the chilli pepper, Capsicum frutescens Mill. Plant Cell, Tissue and Organ Culture 8, 163-176.

Holden, M.A., Hall, R.D., Lindsey, K. and Yeoman, M.M. (1987). Capsaicin biosynthesis in cell cultures of Capsicum frutescens. In Plant and Animal Cells/Process Possibilities, eds. C. Webb and F. Mavituna, pp. 45-63. Ellis Horwood Books.

Lindsey, K. and Yeoman, M.M. (1984). The synthetic potential of immobilized cells of Capsicum frutescens Mill. cv. annuum. Planta 162, 495-501.

Schenk, R.U. and Hildebrandt, A.C. (1972). Medium and techniques for induction and growth of monocotyledonous and and dicotyledonous plant cell cultures. Can. J. Bot. 50, 199-204.

Yeoman, M.M., Miedzybrodzka, M.B., Lindsey, K. and McLauchlan, W.R. (1980). The synthetic potential of cultured plant cells. In Plant Cell Cultures: Results and Perspectives, eds. F. Sala, B. Parisi, R. Cella and O. Cifferri, pp. 327-343. Elsevier/North Holland Biomedical Press.